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SULPHYDRYL-DEPENDENT MONOCYTE-LYMPHOCYTE INTERACTIONS
IN RHEUMATOID ARTHRITIS

Submitted by
M.J. McKeown
for the Degree of Doctor of Philosophy
at the University of Bath
1984

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And still the Weaver plies His loom, whose warp and woof is
wretched Man
Weaving th' unpattern'd dark design, so dark we doubt it owns
a plan.

—The Kasîdah of Hâjî Abdû al -Yazdi

YOUR PROBLEM WILL
BE 'WHAT'S IT ALL ABOUT
AND WHY ARE WE
HERE?'



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CONTENTS

| | Page No. |
|---|----------|
| ACKNOWLEDGMENTS | vii |
| ABBREVIATIONS | viii |
| SUMMARY | x |
| CHAPTER 1: INTRODUCTION | 1 |
| 1.1 RHEUMATOID ARTHRITIS | 2 |
| 1.2 STRUCTURE OF THE SYNOVIAL JOINT | 2 |
| 1.3 PATHOLOGY OF THE RHEUMATOID JOINT | 3 |
| 1.4 EXTRA-ARTICULAR FEATURES OF RA | 6 |
| 1.5 IMMUNOPATHOGENESIS OF RA | 8 |
| 1.5.1 Humoral immunity | 8 |
| 1.5.2 Cell mediated immunity | 10 |
| 1.6 AETIOPATHOGENESIS OF RA | 13 |
| 1.6.1 Immunogenetics | 15 |
| 1.7 IN VITRO STUDIES OF THE IMMUNE RESPONSE IN RA | 16 |
| 1.8 CHEMOTHERAPY IN RA | 18 |
| 1.8.1 Non-steroidal anti-inflammatory drugs | 18 |
| 1.8.2 Second-line antirheumatic drugs | 20 |
| 1.8.3 Gold compounds | 20 |
| 1.8.4 D-Penicillamine | 22 |
| 1.9 THE ROLE OF SH GROUPS IN RA | 25 |
| 1.9.1 Sulphydryl compounds as anti-rheumatic drugs | 25 |
| 1.9.2 Sulphydryl levels in rheumatoid patients | 27 |
| 1.9.3 Immunomodulation by thiol compounds | 28 |

| | |
|---|----|
| CHAPTER 2: MATERIALS AND METHODS | 34 |
| 2.1 MATERIALS AND SUPPLIERS | 35 |
| 2.2 EQUIPMENT AND SUPPLIERS | 36 |
| 2.3 PATIENT SELECTION | 37 |
| 2.4 ROUTINE BUFFERS AND MEDIA | 37 |
| 2.4.1 Phosphate buffered saline (PBS) x 10 | 37 |
| 2.4.2 Calcium magnesium free salt solution (CMFSS) x10 | 38 |
| 2.4.3 Ammonium chloride solution | 38 |
| 2.4.4 Lymphocyte culture medium RPMI 1640 | 39 |
| 2.4.5 Buffers used in the ELISA technique | 40 |
| 2.4.6 Scintillation fluid | 41 |
| 2.5 HUMAN CELL PREPARATION | 41 |
| 2.6 HUMAN CELL CULTURE AND ASSAY TECHNIQUES | 42 |
| 2.6.1 IgG Production | 42 |
| 2.6.2 Lymphocyte proliferation | 43 |
| 2.6.3 Enzyme-linked immunoabsorbance assay (ELISA) for IgG | 43 |
| 2.6.4 Serum SH assay | 44 |
| 2.6.5 Cell surface SH assay | 44 |
| 2.6.6 Blockade of serum SH groups | 45 |
| 2.6.7 Blockade of cell membrane SH groups | 46 |
| 2.6.8 IL-1 Production | 46 |
| 2.6.9 IL-1 Assay | 46 |
| 2.6.10 Production of IL-2 | 47 |
| 2.6.11 Assay for IL-2 activity | 47 |
| 2.7 RAT CELL PREPARATION | 48 |
| 2.8 RAT CELL CULTURE | 49 |

| | |
|---|----|
| CHAPTER 3: RESULTS | 50 |
| 3.1 SYLPHYDRYL-DEPENDENT MONOCYTE-LYMPHOCYTE INTER-ACTIONS IN RHEUMATOID ARTHRITIS | 51 |
| 3.1.1 The role of SH groups in the pathogenesis of Rheumatoid Arthritis | 51 |
| 3.1.2 The role of SH groups in the immune response | 51 |
| 3.1.3 The effect of reducing the available free thiols on mitogen stimulated PBMC proliferation <u>in vitro</u> | 52 |
| 3.1.4 The effect of cell surface SH blockade on PHA-stimulated PBMC proliferation <u>in vitro</u> | 58 |
| 3.1.5 The role of thiols in monocyte accessory cell functions in the immune response <u>in vitro</u> | 62 |
| 3.1.6 Experiment to determine if cell surface SH groups correlate with serum SH levels | 71 |
| 3.1.7 The effect of thiol compounds on IgG synthesis by PBMC from rheumatoid patients | 73 |
| 3.1.8 The role of SH group monocyte-lymphocyte interaction in rheumatoid patients | 77 |
| 3.1.9 Experiments to determine whether blocked surface thiols affect monocyte accessory function in rheumatoid patients | 79 |

| | |
|--|-----|
| 3.1.10 Serial study to determine the effect of D-pen therapy on monocyte accessory function in rheumatoid patients | 85 |
| 3.1.11 Analysis of the action of 2-ME on monocyte-lymphocyte interactions | 94 |
| 3.1.12 Effect of 2-ME on interleukin production | 94 |
| 3.1.13 Studies involving antigen specific rat lymph node proliferation | 96 |
| CHAPTER 4: DISCUSSION | 105 |
| BIBLIOGRAPHY | 118 |

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ABBREVIATIONS

| | |
|----------|---|
| 2-ME | 2-mercaptoethanol |
| 6,6'-D | 6,6' dithiodinicotinic acid |
| BSA | Bovine serum albumin |
| Con-A | Concanavalin A |
| D-pen | D-Penicillamine |
| DTNB | 5,5'-dithiobis (2-nitrobenzoic acid) |
| EBNA | Epstein-Barr nuclear antigen |
| EBV | Epstein-Barr virus |
| ELISA | Enzyme-linked immunoabsorbance assay |
| ESR | Erythrocyte sedimentation rate |
| IgG | Immunoglobulin G |
| IL | Interleukin |
| IL-1 | Interleukin 1 |
| IL-2 | Interleukin 2 |
| LEM | Leucocyte endogenous mediator |
| LY | Lymphocyte |
| MaSF | Active macrophage activated serum factor |
| M ϕ | Monocyte/Macrophage |
| NSAID | Non-steroidal anti-inflammatory drug |
| OMPI | DL-2-oxo-3-(2-mercaptoethyl-5-phenyl-imidazolidine) |
| OVA | Ovalbumin |
| PBM | Peripheral blood monocyte |
| PBMC | Peripheral blood mononuclear cells |
| PG | Prostaglandin |
| PHA | Phytohaemagglutinin |

| | |
|----------|--|
| PHMPSA | p-hydroxymercuriphenylsulphonic acid |
| PMN | Polymorphonuclear leucocyte |
| POPOP | 1,4-Di-2-(4-methyl-5-phenyloxazolyl) benzene |
| PPO | 2,5-Diphenyloxazole |
| PWM | Pokeweed mitogen |
| pro-MaSF | Inactive macrophage activated serum factor |
| RA | Rheumatoid arthritis |
| RANA | RA associated nuclear antigen |
| RF | Rheumatoid factor |
| SH | Sulphydryl |
| SRBC | Sheep red blood cell |
| VCA | Viral capsid antigen |

SUMMARY

The disturbance in thiol expression characteristic of RA was investigated to determine whether it plays a role in the pathogenesis of the disease. This was studied by culturing mononuclear cells in a mitogen-driven system in vitro. Blockage of serum SH groups did not appear to influence mitogenic proliferation of mononuclear cells in vitro. However, mononuclear cell surface SH groups appear to be very much involved in both proliferation and immunoglobulin production. Both cell proliferation and antibody production can be abrogated by blocking the cell surface SH groups. It was shown that rheumatoid mononuclear cells produce less IgG than normal cells when stimulated with pokeweed mitogen in vitro. It was also shown that rheumatoid mononuclear cells will, however, make normal amounts of IgG following the addition, in vitro, of a simple thiol 2-mercaptoethanol (2-ME). Normal IgG synthesis was also observed by mononuclear cells from patients treated with D-penicillamine. The investigation showed that rheumatoid monocytes are defective accessory cells in the IgG production assay and that their function is corrected by 2-ME. However, both monocytes and lymphocytes must be incubated with 2-ME for normal IgG synthesis to occur. We have therefore demonstrated a membrane SH dependent interaction between monocytes and lymphocytes that is necessary for the production of IgG. The interaction does not appear to involve soluble mediators such as the

interleukins or antigen presentation but rather the physical interaction between the lymphocytes and monocytes. This interaction is defective in RA and may be involved in the immunopathogenesis of the disease.

CHAPTER ONE

Introduction

1.1 RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a disease of unknown aetiopathogenesis in which control of inflammatory and immunological processes appears to be aberrant, and which is probably multifactorial in origin (Panayi, et al., 1981). RA is chiefly characterised by an erosive inflammatory polyarthropathy, which classically begins in the small joints of the hands and feet and progresses symmetrically in a centripetal fashion. Any or all of the body's 187 synovial joints may be affected, each presenting different clinical problems due to functional disturbances (Fye et al., 1978; Barnes, 1980).

1.2 STRUCTURE OF THE SYNOVIAL JOINT

Joints that permit free movements are enclosed in a fibrous capsule. The capsule is lined by a serous synovial membrane which also covers the end of each bone, except where it is replaced by articular cartilage. It secretes the synovial fluid which fills the synovial cavity. The synovial fluid acts as a lubricant, permitting free movement of the parts upon one another. The bones of the joint are held in

close alignment by tough connective tissue ligaments (Fig. 1). The synovial membrane consists of two layers - an intimal layer next to the joint cavity and a layer of connective tissue and fat upon which it lies. The intima is predominantly cellular with an abundant blood supply, whereas the subsynovial layer is more fibrous and less vascular (Barnett et al., 1961).

The functions of the cells of the synovial membrane are the production of synovial fluid and the removal of synovial fluid and detritus from the joint space. The joint fluid is essentially a transudate from the blood to which is added hyaluronic acid. Type A cells are active in phagocytosis and secretion. Type B cells are thought to synthesise the hyaluronic acid. In addition there is an intermediate or type AB cell which combines the features of both A and B cells and Ghadially, (1983) has suggested that A and B cells are not different cell types, but variants whose morphology is dependent upon functional activity.

1.3 PATHOLOGY OF THE RHEUMATOID JOINT

In RA the synovial membrane becomes thickened and in the active phases of this disease, exhibits exuberant villus formation. As inflammation proceeds tongues of inflamed villi become adherent to the adjacent margins of the articular cartilage, breaking down the cartilage as it spreads slowly

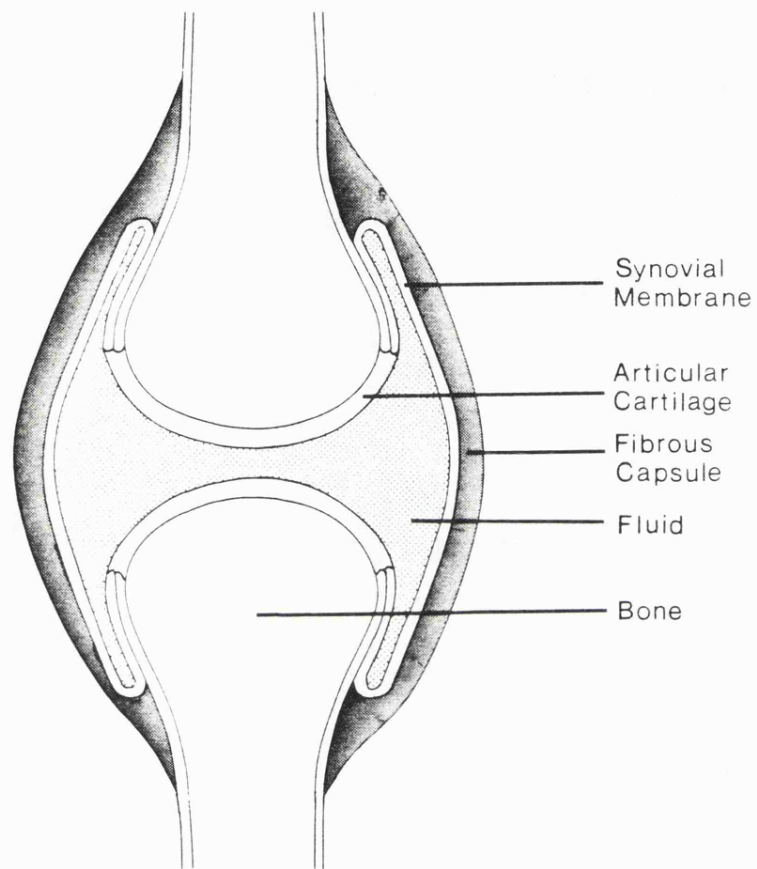


Fig.1 Diagrammatical cross-section showing
the structure of the typical synovial joint.

over it. The tissue, known as pannus, is essentially a sheet of fibro-vascular granulation tissue arising from the perichondral synovial membrane (Barnes, 1980). It is the pannus which is believed to be the source of degradative enzymes which destroy cartilage and bone (Harris, 1976). The articular cartilage becomes increasingly overgrown by pannus which subsequently becomes transformed into relatively acellular mature fibrous tissue, and the resulting fibrous ankylosis leads to increasingly restricted movements in the affected joints (Barnes, 1980).

Articular cartilage is composed of a small number of chondrocytes embedded in a matrix of collagen fibrils entrapped in a ground substance rich in water and proteoglycans. It is the osmotic pressure of the water taken up by the proteoglycans retained and restrained by the collagen network which gives cartilage its resilience and its load bearing properties, (Ghadially, 1983). Shortly after the onset of synovitis, the rheumatoid cartilage has been shown to be subject to proteoglycan depletion as a result of several processes including the release of proteases and glycosidases from Type A cells (Hamerman et al., 1967), the release of similar enzymes by chondrocytes activated by the inflammatory process (Millroy and Poole, 1974), and the release of proteolytic enzymes from polymorphonuclear leucocytes (PMN) and the subsequent intracellular digestion of solubilised proteoglycans within phagolysosomes of these cells (Weissman,

1972). Depletion of proteoglycan from cartilage impairs its ability to rebound after removal of a deforming load, but permanent joint destruction only occurs as a result of collagen loss (Harris, et al. 1972; Harris and Krane, 1974).

Active RA is also accompanied by a great increase in the amount of synovial fluid and an elevation in total cell count. Normal synovial fluid does not clot due to the absence of fibrinogen. In the inflamed rheumatoid synovium fibrinogen passes from the plasma into the joints. There it can be activated to form fibrin. This protein may comprise as much as 34% of the total synovial fluid volume and may be responsible for the development of the classic articular manifestations of RA (Zvaifler, 1973; Jasani, 1979). The cellular composition of the synovial fluid is quite diverse. The majority of the cells (between 75% and 90%), are PMN, the remainder being comprised of 5-10% lymphocytes, some monocytes, macrophages and synovial lining cells, all of which may be involved in the pathogenesis of RA (Zvaifler, 1973).

1.4 EXTRA-ARTICULAR FEATURES OF RHEUMATOID ARTHRITIS

Although arthritis is the most frequent and prominent feature of RA, this disease is a systemic disease affecting many systems of the body. Of the extraarticular manifestations perhaps the most common are subcutaneous nodules which appear in approximately 20-25% of patients.

They are almost invariably associated with seropositive disease, that is, patients whose sera contain autoantibodies to immunoglobulin, which is associated with a more severe and destructive disease (Stage and Mannik, 1973). Areas subjected to mechanical pressure are common sites. Subcutaneous nodules seldom cause symptoms, but occasionally they break down or become infected. Histologically, the rheumatoid nodule is composed of a central area of necrosis surrounded by a palisade of epithelioid cells with variable degree of lymphocyte and plasma cell infiltration and vasculitis. The presence of immunoglobulin and complement in the necrotic areas has been demonstrated but may have little pathogenic significance in such a situation. If this immunoglobulin synthesis occurs in the nodule it may however be relevant to the pathogenesis of the disease (Zvaifler, 1973). Morphological alteration in the structure of the blood vessels or vasculitis also occurs with considerable frequency. This may result from a number of factors, the deposition of immune complexes and fibrin, infiltration of the vessel wall with PMN and mononuclear cells and degradation of collagen and elastin. The clinical effects of vasculitis include skin lesions, peripheral gangrene and visceral lesions leading to dysfunction of the heart, gastro-intestinal tract, kidneys, brain, spinal cord and peripheral nerves (Maini, 1977). Other extra-articular features include muscle wasting, lymph node enlargement, , inflammation and distention of the tendons and bursae, atrophy of the skin, splenomegaly

and leukopaenia. Constitutional symptoms include malaise, fever and weight loss (Fye et al., 1979; Barnes, 1980).

1.5 IMMUNOPATHOGENESIS OF RHEUMATOID ARTHRITIS

Although the underlying reason for the persistent chronic inflammation of the synovial structures and extra-articular tissues in rheumatoid patients remains unknown, an increasing number of observations suggest that both cellular and humoral immunological events mediate its pathogenesis. Microscopic examination of the synovial membrane reveals dense collections of lymphocytes, plasma cells and macrophages in both diffuse and nodular patterns, a picture strongly reminiscent of an antigen stimulated lymph node. (Gardner et al., 1965). Rheumatoid synovium has been referred to as an ectopic lymphoid organ with large numbers of lymphocytes and plasma cells generating immunoglobulin. (Zvaifler, 1973, and Smiley et al., 1968).

1.5.1 Humoral immunity

Many systemic rheumatic diseases are characterised by the presence of one or more serum autoantibodies. Autoantibodies against the Fc fragment of immunoglobulin G molecules were first discovered in the sera of RA patients and hence have been named rheumatoid factors (RF's). RF's may be present in the IgG, IgM and IgA classes (Johnson, 1976). It is now known

that RF's are detectable in the sera of many patients with non-rheumatic diseases and even in some normal individuals (Carson, 1982). In synovial fluid IgG RF predominates (Smiley et al., 1968). IgG anti-IgG may act as both antigen and antibody with the ability to self-associate to form immune complexes (Pope et al., 1974). Self-association of IgG anti-IgG may occur extensively with the resulting complexes providing an inflammatory stimulus within rheumatoid joints and thus playing a major role in synovitis (Munthe and Natvig, 1972; Male and Roitt, 1981). It may be postulated that immune complexes in the synovium could activate the complement cascade. The release of the chemotactic fragments C3a and C5a would promote migration of neutrophils, which together with synovial lining and pannus macrophages, would phagocytose the complexes. The subsequent release of lysosomal enzymes and cationic proteases may contribute to enzymic injury to the articular cartilage and further aggravate the inflammation. The local formation of IgG anti-IgG complexes would make the rheumatoid joint akin to an Arthus reaction (Maini, 1977). Immune complexes may also form in the bloodstream and subsequently localise in tissues. Such a pattern is seen in the acute and chronic models of serum sickness. Maini (1977) has suggested that the presence of complexes in the bloodstream might contribute to the multi-system features of RA, as well as vasculitis. Antibody synthesis is known to be controlled, at least partially, by feedback inhibition. The complexing of IgG RF may impair the negative feedback

regulation of IgG synthesis in the plasma cells and could explain why they produce IgG RF at an apparently uncontrolled rate, leading to self-perpetuating immunologic and inflammatory processes (Natvig and Munthe, 1975). There is a good deal of evidence to support the suggestion that immune complexes are involved in the pathogenesis of RA. Complement component levels are reduced in synovial fluids in a majority of rheumatoid patients and are inversely correlated with immune complexes (Lunthra et al., 1975). Deposits of immune complexes have also been demonstrated in acutely inflamed vessel walls (McDuffie, 1978). Further evidence includes the localisation of immunoglobulin and complement in articular cartilaginous tissue of most RA patients (Cooke et al., 1975) and the presence of large inclusion bodies containing immunoglobulin and complement in the PMN of patients with Felty's syndrome (Andreis et al., 1978). A strong correlation between raised levels of IgG RF and vasculitis has been noted (Allen et al., 1981; Scott et al., 1981). In the latter study patients were found to have circulating complexes with anti-complementary activity, and serum C4 levels below the normal range, which indicated that the IgG-RF complexes bound complement.

1.5.2 Cell mediated immunity

The role of cell-mediated tissue injury may be no less significant than that which may be caused by antigen-antibody

complexes. Indeed, RA can occur in congenitally agammaglobulinaemic boys, where complexes presumably play no role at all (Taussig, 1979). There is also a predominance of T cells in the rheumatoid synovial membrane and T cell derived lymphokine like activity has been demonstrated in synovial fluids and supernatants of explants of inflamed rheumatoid synovial tissue (Stastny et al., 1975).

Lymphokines are soluble factors produced by the mononuclear cells that act as regulatory signals which modulate the activity of cells participating in the immune, inflammatory and reparative processes. It is not clear how many lymphokines there are since many appear to have multiple biological effects. The Second International Lymphokine Workshop (1979) made an attempt to define molecular entities rather than biological activities, which lead to the introduction of the term interleukins (IL). IL-1 and IL-2 were distinguished by their biochemical properties and biological activities. Human IL-1 was defined as a macrophage-derived factor with a MW of between 12,000 and 15,000 which enhanced lymphocyte proliferation while IL-2 was defined as a factor of 30,000MW which could support the continuous proliferation of cytotoxic T-cells - the inability of IL-1 to act as a growth factor for cytotoxic T-cells was the strongest evidence that IL-1 and IL-2 were different molecules. Larsson (1982) has suggested that these definitions were premature and Oppenheim and Gery (1982) have

proposed that IL-1 possesses too many biological effects to be a single molecule. These workers have shown that IL-1 has important actions on several cell types in addition to lymphocytes. IL-1 appears to be similar if not identical to endogenous pyrogen and leucocyte endogenous mediator (LEM) (Sztein et al., 1981). LEM triggers the synthesis of acute phase proteins by hepatocytes (Kampschmidt, 1978). Several connective tissue cell types also respond to IL-1. Synoviocytes are stimulated to secrete collagenase and prostaglandins (Mizel et al., 1981), fibroblasts proliferate (Schmidt et al., 1981) and chondrocytes are activated to degrade the cartilage matrix (Jasin and Dingle, 1981). Thus IL-1 induces many cell functions that are relevant to the inflammatory process in general (fever, acute phase response) and to connective tissue in particular (synoviocytes, fibroblasts, chondrocytes activation). Any defect in the regulation of these cells could therefore lead to the immune and inflammatory aberrations characteristic of RA.

The pathogenic role of lymphoid cells in RA is emphasised by the achievement of clinical improvement with the use of treatments which are known to be immunosuppressive. Removal of immunoglobulins and immune complexes from plasma using plasmapheresis (Lockwood, 1979), the removal of immunocompetent lymphoid cells by thoracic duct drainage (Paulus et al., 1977), and total lymphoid irradiation (Kotzin et al., 1981), frequently lead to dramatic clinical

improvement. Together with these physical means of suppressing lymphocyte function the steroidal drugs, which have a wide range of actions including immuno suppression, are extremely potent anti-inflammatory agents (Hart, 1979). These treatments may however defeat the object of the immune response and render the patient susceptible to many environmental pathogens.

1.6 AETIOPATHOGENESIS OF RHEUMATOID ARTHRITIS

The principal immunopathogenic features of RA may be due to a defect in immunoregulation either in isolation or related to a persistent antigen (Allison, 1977). Candidates for this initiating antigen include infectious agents, aggregated IgG and components of synovial tissue, cartilage and collagenous tissues (Paget and Gibofsky, 1979). Although an infectious cause for RA remains attractive, there is no consistent microbiological evidence available. However, bacteria are known to be the causative agents in reactive arthritis. This explosive monoarthritis, which occurs as a consequence of a distal infection, can be resolved with antibiotic therapy (Munoz, 1978). Similar changes to those occurring in the synovium and synovial fluid in RA are seen in Lyme disease (Steere et al., 1977). Immune complexes are also present in this systemic inflammatory disorder, although RF is absent (Hardin et al., 1979). Strong circumstantial evidence favours transmission of the causative agent by a minute tick,

Ixodes dammini (Steere and Malawista, 1979). A spirochete has recently been isolated from these ticks and is under study as a potential aetiologic agent (Burgdorfer et al., 1982). Oral penicillin G or tetracycline may prevent or attenuate the subsequent arthritis (Steere et al., 1980). More attractive candidates for the causative agent of RA are viruses. The

arthritis caused by viruses mimics some of the symptoms of RA. Onset is characterised by fever and malaise, the arthritis is usually symmetrical and affects the same spectrum of joints as RA. The major difference between the two disease states is that reactive arthritis is transitory and self-limiting (Van Sauter and Utsinger 1978). A possible association between RA and Epstein-Barr virus (EBV) has been proposed. EBV is a polyclonal B cell activator which is ubiquitous in the environment (Rosen et al., 1977). Alspaugh and Tan (1976) and Alspaugh et al. (1978) have demonstrated that the sera of approximately 65% of RA patients react with cell associated nuclear antigen present in Wil2 human lymphoid cell lines induced by EBV infection compared to low levels of the antibody in normal healthy controls. This RA associated nuclear antigen (RANA) appears to be distinct from Epstein-Barr nuclear antigen (EBNA) (Alspaugh et al., 1978). Catalano et al., (1980) studied healthy individuals and showed that those having antibodies to viral capsid antigen (VCA), which is expressed during EBV infection, also had anti-RANA antibodies, while anti-VCA negative individuals were anti-RANA negative. They concluded, therefore, that anti-RANA

antibodies are produced only after EBV infection. Lymphocytes from both normal and RA individuals can be stimulated in vitro to produce IgM RF by EBV infection (Slaughter et al., 1978). A similar production of IgM RF following injection of bacterial lipopolysaccharide in mice has been reported (Dresser, 1978). It may be proposed that naturally occurring B cell activators, such as EBV, may initiate the stimulation of clones of cells in vivo which would react with autoantigens and result in elevated levels of autoantibodies including RF. Any involvement of EBV in the pathogenesis of RA must however remain speculative (Depper and Zvaifler, 1981), particularly when the polyclonal nature of this stimulus is compared with the restricted spectrum of autoantibodies, largely antiglobulins, detected in RA patients.

1.6.1 Immunogenetics

The difficulty in determining initiating factors may be due to the possibility that such factors are environmental agents which do not normally induce disease but which in genetically susceptible individuals may do so by interacting abnormally with the immune system. The interest in the influence of genetics on the aetiopathogenesis of rheumatic diseases has been stimulated by the work of Brewerton et al. (1973), who demonstrated a strong association between ankylosing spondylitis and the presence of HLA-B27. A relatively high risk of predisposition to RA has been observed

in HLA-DR4+ compared to DR4- individuals (Stastny, 1978; Panayi et al., 1976). The precise links between these genetic markers and the pathogenic mechanisms underlying each disease are still unclear. It has recently been suggested (Jones et al., 1983) that HLA-DR4 is a marker of likely disease severity in RA rather than of susceptibility to arthritis. This is consistent with the observation that patients with extra-articular manifestations of severe RA are almost all HLA-DR4W positive (Dinant et al., 1980).

1.7 IN VITRO STUDIES OF THE IMMUNE RESPONSE IN RHEUMATOID ARTHRITIS

Much of the research aimed at determining whether or not a defect in immunoregulation exists in RA has been directed towards the peripheral blood mononuclear cells (PBMC). These cells are more readily available from both healthy controls and rheumatoid patients than synovial tissue. Although there are many conflicting reports it is generally accepted that normal T- and B-lymphocyte proportions exist in the peripheral blood of rheumatoid patients (Paget and Gibofky, 1979). Earlier studies of cellular immunity based on skin testing to common antigens showed significant abnormalities in patients with RA. Waxman et al. (1973) found that 42% of RA patients were completely anergic to five common antigens. Another common estimate of the functional status of PBMCs used in recent years has been cell proliferation in response to a

mitogens such as concanavalin A(ConA) or phytohaemagglutinin (PHA). Studies of the mitogenic response of PBMCs from RA patients have not revealed consistent results from one laboratory to another. Some investigators have reported no significant differences between rheumatoid patients and controls (MacLaurin 1971; Reynolds and Abdou, 1973; Kinsella, 1974). Others have noted some very low responses among RA patients but have reported normal mean values (Denman et al., 1973; Kacaki et al., 1969; Waxman et al., 1973.). Still others have reported generally low responses in RA (Griswold and McIntosh, 1973; Lance and Knight, 1974; Lockshin et al., 1975; Highton et al., 1981). These discrepant reports might be explained by the heterogeneity of the patients studied and differences in the methodological approach of various investigators. It has been proposed that there are indeed decreased responses in patients with RA but this response is limited to a subset of patients characterised by the presence of erosive joint disease (Silverman et al., 1976). In more recent years lymphocyte proliferation assays have been superseded by in vitro pokeweed mitogen (PWM) stimulated production of immunoglobulin, measured using an enzyme-linked immunosorbance assay (ELISA). A number of workers have reported reduced levels of immunoglobulin production by PBMCs obtained from RA patients compared to healthy controls (Alarcon et al., 1982; Poikonen et al., 1982; Plater-Zyberk et al., 1983). The anergic reactions of RA patients to common antigens and the hyporesponsiveness of rheumatoid mononuclear

cells in culture may seem a paradox considering the nature of the disease. RA is a disease in which the immune response is hyperactive, and one might expect the opposite to occur. Possible explanations for this depressed immune response in RA include preoccupation of the immune mechanisms of the host with reactivity related to the pathogenesis of the disease, viral infections or immune complexes.

1.8 CHEMOTHERAPY IN RHEUMATOID ARTHRITIS

1.8.1 Non-steroidal anti-inflammatory drugs

The mode of action of many of the drugs used in RA may involve the manipulation of one or more components of the immune system. As has been previously stated, treating RA by simply suppressing the immune system is undesirable. Lacking a cure for RA any treatment must be empirical and this is initially to treat the symptoms. The non-steroidal anti-inflammatory drugs (NSAID) do not cure or permanently reverse the inflammatory process in any arthropathy, but by reducing swelling and pain and allowing better joint function, do help the clinical condition considerably (Hart et al., 1981). NSAID are to a greater or lesser degree anti-inflammatory analgesic and antipyretic. Aspirin and the salicylates are the most widely used of the NSAID and have been used in medicine since 1899. Indomethacin and phenylbutazone followed and more recently large numbers of drugs based on different

organic acids such as propionate and phenylacetate. Vane (1971) proposed that the NSAID act as anti-inflammatory agents by inhibiting prostaglandin (PG) synthesis. They interact directly with the enzyme, cyclooxygenase and inhibit the synthesis of all PGs. In vitro all NSAID are active inhibitors of cyclooxygenase (Crook et al., 1976). As well as their beneficial effects they may also produce toxic side effects on the gastrointestinal tract. PGs promote pain (Ferreira, 1978), fever (Feldberg et al., 1972), swelling (Wedmore and Williams, 1981) and may have a protective effect on the gastric mucosa (Rainsford, 1982), all of which appears to support Vane's hypothesis. However, while clinical experience suggests that, in general, the NSAID are equally effective in patients, their potencies as cyclooxygenase inhibitors vary widely (Crook et al., 1976). Salicylate, which has little effect on PG synthesis, is clinically indistinguishable from aspirin. Also, cyclooxygenase activity in rheumatoid synovial tissue may be abolished in patients taking low doses of aspirin (600 mg day^{-1}) which do not exert detectable antiinflammatory effects. PGs are a family of compounds and have also been shown to have anti-inflammatory (Kunkel et al., 1981) and immunosuppressive (Goodwin et al., 1977) effects. PGs can modulate each other directly and are also self-modulating via the cyclic nucleotides (Dunn et al., 1976). The PGs also interact with other mediators of inflammation (Trang, 1980). It is unlikely, therefore, that suppression of PGs is the unique site of action of the NSAID.

Moreover, it is perhaps unwise to block completely the production of such an important group of physiological mediators.

1.8.2 Second-line antirheumatic drugs

NSAID merely treat the symptoms leaving the long term course of the disease unchecked. If the disease does progress another group of drugs variously termed second line, antirheumatic, disease modifying, remission inducing, may be used. These agents differ from classical anti-inflammatory drugs in that they appear to have the capacity to modulate the course of RA and, in some cases, to induce remission of the disease. The most successful anti-rheumatic agents represent the outcome of shrewd clinical observations in the use of compounds introduced for other diseases rather than the result of systematic research on the part of the pharmaceutical industry.

1.8.3 Gold compounds

In the early 1920s the similarity between RA and tuberculosis and the ability of gold to inhibit the growth of tubercle bacilli in culture prompted physicians to treat rheumatoid patients with gold salts that had been employed, erroneously, in the management of tuberculosis (Bird and Wright, 1982). That gold salts have survived since their

introduction in the 1920s may be seen as either a tribute to their effectiveness, or a reflection of the absence of a truly antirheumatic drug. A number of controlled clinical trials have established that gold compounds are effective in the treatment of RA (Research Sub-committee of the Empire Rheumatism Council, 1960; Research Sub-committee of the Empire Rheumatism Council, 1961; Co-operating Clinics Committee of the American Rheumatism Association, 1973) and can modify the course of the disease as judged radiologically. The mode of action of gold compounds however, remains unknown.

Aurothiomalate has been shown to inhibit several lysosomal and other cellular and extra-cellular enzymes (Persellin and Ziff, 1966; Janoff, 1970; Naccache et al., 1977). These enzymes may be inhibited intracellularly in vivo, as elemental gold is concentrated in the reticuloendothelial system (Gottlieb et al., 1972) and may be readily detected within phagocytic cells in rheumatoid synovial tissue (Vernon-Roberts et al., 1976; Nakamura and Igaraski, 1977). In vitro the effects of gold compounds on the immune system have been investigated in several assay systems. Aurothiomalate has been shown to inhibit complement activation in vitro (Schutz et al., 1974; Burge et al., 1978). Both these effects are mediated by the gold component of the drug. The actions of gold compounds on mononuclear cell function are more complex. In vitro, gold has been shown to suppress antigen and mitogen driven lymphocyte proliferation (Lipsky and Ziff,

1977; Lies et al., 1977). Lipsky and Ziff (1977, 1982) have proposed that this effect is caused by inhibition of monocyte accessory function in the culture system. These observations in vitro contrast with studies carried out with PBMC from RA patients being treated with aurothiomalate, in whom a good clinical response is accompanied by a change in lymphocyte responsiveness from a depressed state to normal (Davis et al., 1979, Highton et al., 1981).

1.8.4 D-Penicillamine

On equally slender and highly empirical grounds, D-penicillamine (D-pen) has been similarly used in the treatment of RA. D-pen is 2,2'-dimethyl cysteine, a degradation product of penicillin, and named penicillamine by Abraham and associates (1943). D-pen is used in the treatment of three unrelated disorders, Wilson's Disease, cystinuria and RA (Lyle, 1979). The usefulness of D-pen in the treatment of patients with RA has been well established (Multicentre Trial Group, 1973). Its actions are slow and selective since it does not benefit all types of inflammatory arthritis. D-pen exerts its effects on extraarticular features of the disease, as well as on the joints, and there is often reduction in RF and erythrocyte sedimentation rate (ESR). In patients able to continue treatment, there is improvement in the outcome of the disease as judged by delay in radiological progression. The mode of action of the drug is unknown. It was first used

because of its ability to reduce titres of RF (Jaffe, 1970), but this is almost certainly not its mode of action. In vitro studies by other investigators have demonstrated the capacity of this compound and other thiols such as 2-mercaptoethanol (2-ME) to dissociate macroglobulins by cleavage of their inter-subunit disulphide bonds. It is however unlikely that the reduction of circulating RF titres results directly from the action of D-pen, since the concentration of D-pen attained in vivo is orders of magnitude less than that needed to dissociate macroglobulins (Van de Stadt et al., 1979). These in vitro studies do however illustrate one important aspect of D-pen's interactions with body tissue, namely sulphydryl disulphide exchange.

Another feature of D-pen is its ability to chelate metal ions, including copper (Lyle, 1979). These observations explain the use of this drug in treating cystinuria and Wilson's disease, but their significance for the actions of D-pen in RA remains obscure. D-pen enhances serum sulphydryl-disulphide interchange reactions (Hall and Gillan, 1979) and dissociates IgA-alpha, antitrypsin complexes in vivo (Wollheim et al., 1979). In vitro D-pen inhibits the sulphydryl dependent heat denaturation of IgG (Gerber, 1978) and might therefore prevent the formation of autoantigenic IgG aggregates in vivo. The scavenging of free radicals, associated with the formation of aggregated IgG by D-pen may result in a similar effect (Wickens et al., 1983). Copper-D-

pen complexes have been shown to express superoxide dismutase-like activity, although at relatively high concentrations (Greenwald, 1981). Thiol compounds, including D-pen, have been shown to be potent inhibitors of myeloperoxidase activity (Matheson, 1982) which could contribute to protection of tissue from oxidative damage (Rosen and Klebenoff, 1979).

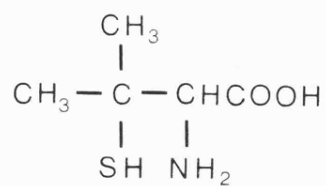
In-vitro studies involving the effects of D-pen on cell function have often produced conflicting and ambiguous results. This is probably due largely to the difficulty in determining a realistic level of the drug due to use in culture. Chwalinska-Sadowska and Baum (1976) have reported inhibition of chemotaxis by D-pen whereas Mowat (1978) has failed to demonstrate this effect. D-pen has also been reported to have a biphasic effect on lymphocyte responses to mitogens, with "low" doses enhancing the responses and higher doses resulting in suppression (Maini and Roffe, 1976). Lipsky and Ziff (1982) have shown that whilst D-pen alone causes a modest decrease in mitogen-induced lymphocyte proliferation the addition of copper salts to D-pen containing cultures results in a marked augmentation of the degree of inhibition. Thus, whilst an immunomodulatory effect of D-pen seems likely, exactly what that effect is remains unclear.

1.9 THE ROLE OF SH GROUPS IN RHEUMATOID ARTHRITIS

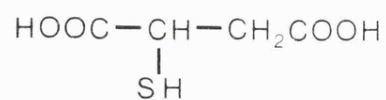
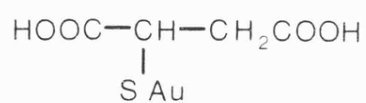
1.9.1 Sulphydryl compounds as anti-rheumatic drugs

It is interesting to note that many of the second line antirheumatic drugs are either thiol compounds or are converted to thiol compounds in vivo (Hunneyball, 1980). D-pen is a thiol and Munthe (1978) has observed that all the gold salts examined in controlled long term studies have been thiols. Levamisole is an antirheumatic compound which has been shown to possess second-line antirheumatic actions in double-blind studies (Multicentre Study Group, 1978, Symoens and Schuermans, 1979). The drug is now rarely used in RA because of toxic side effects (Symoens et al., 1979). Levamisole has a number of effects on mononuclear cell function both in vitro and in vivo that has led to its being called "immunorestorative" and "thymomimetic" (Symoens and Rosenthal, 1977). Its clinical and biological effects are similar to those of gold and D-pen (Symoens and Schuermans, 1979). Levamisole is extensively metabolised in vivo, a major product being DL-2-oxo-3-(2-mercaptoethyl-5-phenyl-imidazolidine) (OMPI) which contains a free thiol group (De Brabander et al., 1978). Jaffe (1980) has suggested that these sulphydryl (SH) compounds are the first of a new generation of anti-rheumatic drugs and that their effectiveness may be due, at least in part, to their SH moiety.

D-penicillamine

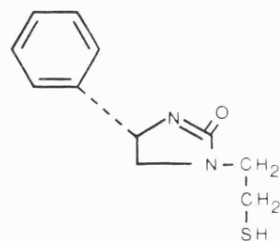
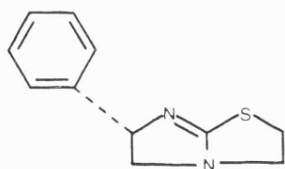


Aurothiomalate



Au-S-Protein

Levamisole



OMPI

Fig. 2. Structural formulae of Auranofin, D-pen and Levamisole.

1.9.2 Sulphydryl levels in rheumatoid patients

Abnormally low SH levels are found in RA serum (Lorber et al., 1964) and serial measurements show an inverse correlation with disease activity (Haataja, 1975). A similar depression in erythrocyte cell surface SH groups has been shown to correlate with serum SH levels (Lorber and Chang, 1968). It is not clear whether this disturbance in thiol expression plays a role in the pathogenesis of RA. However, Hall et al. (1981) have shown that following treatment with D-pen and aurothiomalate, serum SH levels rose to normal values in good clinical responders.

In plasma the vast majority of SH groups are present on the albumin molecules and the normal albumin thiol concentration is about 0.7 thiols per albumin molecule (Foster, J.F., 1977). The reason for this non-integral number arises because some thiols are blocked by the formation of a disulphide bond between the albumin thiol and small SH compounds such as cysteine, and the depression of serum SH in RA appears to be due to further oxidation and subsequent mixed disulphide formation (Thomas and Evans, 1975). Hall et al., (1984) has proposed that free radicals produced during the inflammatory processes are responsible for promoting this reaction. During acute inflammation there is a general alteration of liver protein synthesis, the acute phase response, and a decrease in albumin synthesis occurs in order

to allow for the rapidly increased synthesis of a number of substances, collectively known as the acute phase reactants, that play an essential role in host defence (Sipe and Rosenstreich, 1981). This decrease in albumin concentrations is obviously a partial cause of decreased thiol levels in RA, but the lack of a good correlation between albumin and thiol concentrations suggests it is not a major effect (Banford et al., 1982). Caeruloplasmin is an acute phase reactant which rapidly oxidises some low molecular weight thiols such as cysteine and thiomalic acid to disulphides (Albergoni and Cassini, 1978). It does not affect the thiol of albumin directly presumably because of steric hindrance between the two proteins. However, low molecular weight disulphides exchange readily with the thiol of albumin (Foster, 1977) and therefore, the production of disulphides by caeruloplasmin could be a determining factor in the reduced thiol concentration. Alternatively, or in addition to this, the release of hydrogen peroxide singlet oxygen, and hydroxyl radicals, from stimulated neutrophils and macrophages, may play a role in the decreased SH levels (Badwey and Karnovsky, 1980; Hall et al., 1984).

1.9.3 Immunomodulation by thiol compounds

Whatever the cause of the blocked serum and cell surface SH groups in RA it may have a profound effect on the

pathogenesis of the disease. SH groups are a highly reactive species with important functions in several biological processes including inflammation and the immune response (Jensen, 1959). In animal studies, several aspects of the immune response have been shown to involve SH groups. Simple thiols such as 2-ME has been shown to potentiate the function of many immunological cells in vitro, including antibody responses (Click et al., 1972), lymphocyte proliferation (Broome and Jeng, 1973; Bevan et al., 1974), cytotoxic T-cell responses (Cerottini et al., 1974; Harris et al., 1976; Igarashi et al., 1977) and the growth of B-lymphocyte colonies (Metcalf, 1976). Click et al. (1972) found that the addition of 2-ME at an optimal dose of $2 \times 10^{-5}M$ resulted in a marked increase in the plaque forming cell response of unfractionated spleen cells. These authors concluded that this effect was not due to cell viability because the reagent did not need to be added daily to the culture. Rather, they postulated that 2-ME was exerting its effects on a very early event since the half-life of the reducing agent was very short. Chen and Hirsch (1972) examined the action of 2-ME on the plaque forming cell response of spleen cells depleted of adherent cells. They showed that the depletion resulted in a marked reduction of the antibody response and that the addition of peritoneal macrophages or 2-ME would reconstitute it. It is therefore often stated that 2-ME can substitute functionally for macrophages. The critical point is a question of whether the response of T- and B-cells to SRBC in the presence of 2-ME

is truly macrophage independent. Hodes and Singer (1977) showed that when accessory cells were functionally depleted by adherence using Sephadex G-10 columns, there was a strict dependence of accessory cells without any effect whatsoever of 2-ME. These results confirm those of Erb and Feldmann (1975abc). Rosenstreich and Mizel (1978) demonstrated that 2-ME enhanced the activity of very small numbers of macrophages added to the culture and concluded that 2-ME synergised with macrophages to increase the response.

It is not known whether in the above systems 2-ME is acting directly on the cell or through a secondary product. Broome and Jeng (1973) made a very thorough analysis of the effect of various thiols on the growth of normal spleen cells and various lymphoid tumour lines. Normal spleen cells and 13 of 22 tumour lines had increased growth in cultures when the various thiols were added. In contrast, other cell lines including HeLa and fibroblasts were not affected by 2-ME. Reactions involving SH groups can therefore markedly affect both lymphocytes and monocytes.

The growth of the mouse lymphoma cell line L12.10/F10.30 is dependent on the presence of either a thiol or macrophages. This lymphoma may represent cells that have retained some of the regulatory mechanisms of the normal lymphocyte (Hewlett et al., 1977). This would suggest that the mode of action of macrophages in its accessory role is, at least in part, via

the SH group. Experimental evidence has shown that 2-ME or macrophages do not act directly on the lymphoma cells but on a serum factor. The macrophage activated serum factor (MaSF) is thought to be albumin (Hewlett, personal communication, 1981). Albumin is known to be essential for the growth of lymphocytes in culture (Polet and Spieker-Polet, 1975; Spieker-Polet and Polet, 1976; Arai et al., 1977). Hewlett (personal communication, 1981), proposed that MaSF exists in two forms, an inactive form (pro-MaSF) and an active form (MaSF). They postulated that pro-MaSF was albumin and MaSF mercaptalbumin. The addition of 2-ME, and presumably macrophages, reduced the albumin to mercaptalbumin, which they suggested was necessary for the immune response to take place (Fig. 3). MaSF thus provides one mechanism whereby macrophages may modulate the immune response via an SH reaction. It is not known whether these observations have any relevance to normal immune responses in humans. It may be speculated that if it does, the blocked SH groups present in RA contribute to the abnormalities in the immune response in this disease. In experiments with normal lymphocytes in culture, there is considerable evidence to suggest that the effects of low molecular weight thiols on lymphocyte activity are mediated by cell surface SH groups and not by intracellular thiols or by secondary mediators. Glutathione, an impermeant thiol, has been shown to enhance lymphocyte proliferation in a similar manner to 2-ME, whereas cysteamine phosphate, a compound containing no free SH groups until it is dephosphorylated

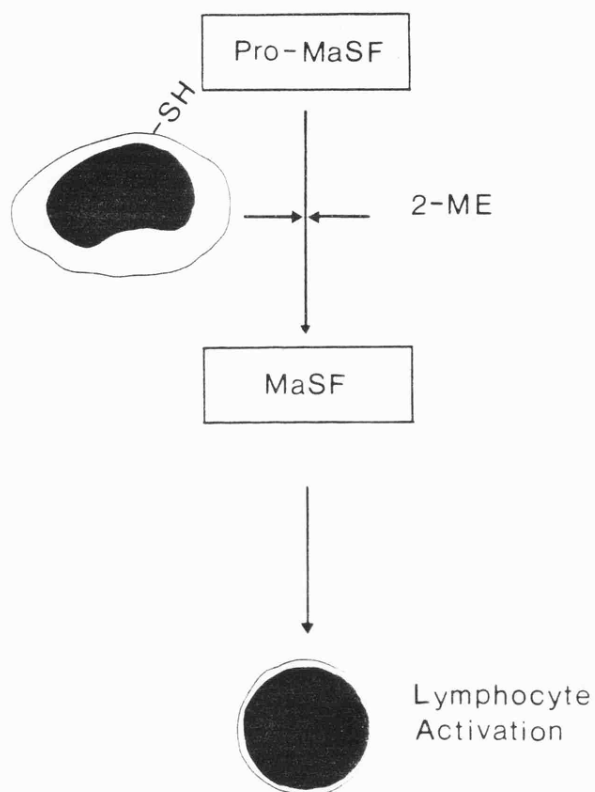


Fig. 3. Flow diagram to illustrate the MaSF hypothesis. Pro MASF is activated to MaSF by its oxidation - either by a thiol compound or a m ϕ . MaSF is necessary for activation of the lymphocyte.

within the cell, does not (Noelle and Lawrence, 1979). Furthermore, it has been demonstrated that lectin-induced cell proliferation can be inhibited by blocking the free SH groups on the cell surface (Chaplin and Wedner, 1978).

Abnormally low serum SH levels are a feature of active RA and following their successful treatment with sulphydryl drugs the SH levels return to normal. The immune system is intimately involved in the pathogenesis of RA, and can be influenced by thiols. The aim of this project was to determine the possible role of SH groups in immunoregulation and to determine whether they might be involved in the immunopathogenesis of RA. In particular it was hoped to identify the ways in which D-pen might modulate the immune response in RA patients, and thereby exert both its anti-rheumatic actions and perhaps its toxic side effects.

CHAPTER TWO

Materials and Methods

2.1 MATERIALS

SUPPLIER

All reagents and disposable tissue culture plastics, except where otherwise stated:

Gibco Bio-cult Limited,
Paisley, Scotland.

All chemicals, except where otherwise stated:

Sigma Chemical Company
Limited, Poole.

Ficoll-paque, Percoll,
Sephedex G-10, Sepharose 6B:

Pharmacia (GB) Limited,
Milton Keynes.

IL-1 and IL-2 standards:

Koch-Light Limited, Suffolk.

Scintillation grade toluene:

British Drug Houses (BDH)
Limited, Poole.

Scintillation vials:

Richardsons of Leicester,
Leicester.

Visking dialysis tubing:

Medicell International
Limited, London.

2.2 EQUIPMENT

SUPPLIER

| | |
|---|---|
| MSE Chilspin 2 Centrifuge: | MSE Scientific Instruments, Sussex. |
| Skatron cell harvester: | Flow Laboratories Limited, Irvine, Scotland. |
| Laborlux 12 microscope: | E. Leitz (Instruments) Limited, Luton. |
| Elisa reader: | Gilford Instruments, Ohio, USA. |
| Packard Tri-carb scintillation counter 3255: | United Technologies, Packard, Berkshire. |
| CE292 digital ultraviolet spectrophotometer: | Cecil Instruments, Cambridge. |

2.3 PATIENT SELECTION

Patients were assessed by either Dr. P. Maddison or Dr. A. Woolf. Patients designated RA-NSAID had sero-positive disease with active sinovitis and were receiving NSAID therapy only. Patients designated RA-D-pen were those who responded well to D-pen therapy. Healthy controls were age and sex matched with patients.

2.4 ROUTINE BUFFERS AND MEDIA

2.4.1 Phosphate buffered saline (PBS) x 10

| | gdm ⁻³ |
|--------------------------------|-------------------|
| Sodium chloride | 80.00 |
| Potassium chloride | 2.00 |
| Disodium hydrogen phosphate | 11.50 |
| Potassium dihydrogen phosphate | 2.00 pH 7.3 |

The above were dissolved in 1 dm⁻³ distilled water, filter sterilised (Millipore filter 0.45 µm pore size) and stored at ^{4°C} until required.

Before use, the solution was diluted to ten times its volume with distilled water. To this was added the equivalent of:-

0.1 g.dm⁻³ calcium chloride

0.1 g.dm⁻³ magnesium chloride

The pH was corrected to 7.3 with 1M sodium hydroxide solution.

2.4.2 Calcium magnesium free salt solution (CMFSS) x10

| | gdm ⁻³ |
|--------------------------------|-------------------|
| Sodium chloride | 80.00 |
| Potassium chloride | 4.00 |
| Glucose | 16.00 |
| Potassium dihydrogen phosphate | 0.60 |
| Disodium hydrogen phosphate | 0.48 pH 7.3 |

The above were dissolved in 1 dm³ distilled water, filter sterilised, and stored at 37°. Before use, a 10 fold dilution was performed with distilled water and the pH corrected to 7.3 with 1M sodium hydroxide.

2.4.3 Ammonium chloride solution

This solution was used for the lysis of extraneous red cells in the rat spleen cell suspension.

gdm⁻³

| | |
|--------------------------------|-------|
| Ammonium chloride | 8.290 |
| Potassium hydrogen carbonate | 1.000 |
| Ethelenediaminetetracetic acid | 0.004 |

The above constituents were dissolved in 1 dm³ distilled water and the pH corrected to 7.4 with 1M sodium hydroxide.

2.4.4 Lymphocyte culture medium RPMI 1640

Volume (cm³)

| | |
|--------------------------------------|-------------|
| RPMI 1640 (x10 strength) | 10.00 |
| Penicillin and Streptomycin solution | |
| 5000IU cm ⁻³ of | 2.00 |
| 200 mM glutamine | 2.00 |
| 7.5% sodium bicarbonate | 2.70 pH 7.4 |
| Foetal calf serum | 10.00 |

The above mixture was diluted to 100 cm³ with sterile, distilled water. The pH was corrected to 7.4 with sterile 1M sodium hydroxide.

2.4.5 Buffers used in the ELISA technique

Coating buffer

| | g dm^{-3} |
|--------------------|--------------------|
| Sodium carbonate | 1.59 |
| Sodium bicarbonate | 2.93 |
| Sodium azide | 0.20 pH 9.6 |

The constituents were dissolved in sterile, distilled water and the solution stored at room temperature for not more than two weeks.

PBS/Tween

| | |
|---|--------------------|
| Tween 20 (Polyoxyethylene sorbitan monolaurate) | 0.5 cm^3 |
| Sodium azide | 0.2 g |

The above constituents were added to 1 dm^3 PBS and the buffer stored at room temperature.

Diethanolamine buffer (10%)

| | |
|--------------------------------|--------------------|
| Diethanolamine | 97 cm^3 |
| Distilled water | 800 cm^3 |
| Sodium azide | 200 mg |
| Magnesium chloride hexahydrate | 100 mg |

The above constituents were mixed and adjusted to pH 9.8 with 1M hydrochloric acid. The total volume was made up to 1dm³ with distilled water and the buffer stored in an amber bottle at room temperature.

2.4.6 Scintillation fluid

To 2.5 dm³ of toluene was added:-

12.50 g 2,5 - Diphenyloxazole (PPO)

0.75 g 1,4 - Di-2-(4-methyl-5-phenyloxazolyl)

benzene (POPOP)

2.5 HUMAN CELL PREPARATION

PBMC were prepared from heparinised peripheral blood, diluted with an equal volume of CMFSS, and by density flotation on Ficoll-Paque (Boyum, 1968). 7 cm³ of diluted blood was layered onto 3 cm³ of Ficoll-Paque in sterile plastic centrifuge tubes. The tubes were then centrifuged at ^{400g} for 30 minutes. The PBMC was removed from the interface, washed three times with CMFSS and resuspended in 1 cm³ RPMI 1640. The cells were counted in an haemocytometer and then diluted with RPMI 1640 to give 10⁶ cm⁻³. In some experiments PBMC were further separated into monocyte and lymphocyte-enriched fractions by centrifugation through 52% v/v Percoll. PBMC in 1 cm³ of RPMI 1640 were layered onto 2 cm³

of 52% Percoll in sterile plastic centrifuge tubes. The tubes were centrifuged at ^{750g} for 30 minutes. After centrifugation, monocytes (greater than 90% pure by morphology) were obtained from above the Percoll layer and lymphocytes from the pellet. The latter were depleted of any residual monocytes by passage through a 10 ml column of Sephadex G10 (Pharmacia) equilibrated with CMFSS containing 5% FCS. The various cell populations were washed three times with CMFSS and resuspended in RPMI 1640 and counted. All tissue culture experiments were carried out using aseptic techniques.

2.6 HUMAN CELL CULTURE AND ASSAY TECHNIQUES

2.6.1 IgG Production

The PBMC, monocytes and lymphocytes were cultured alone or in various combinations using standard conditions. PBMC or lymphocytes (10^6 cm^{-3}) were cultured in 0.2 cm^3 aliquots of RPMI 1640 containing 10% FCS in flat-bottom microtitre plates. Incubations were set up in the absence or presence of PWM (final dilution 1:1000). After culture for 14 days at 37C in a humidified atmosphere of 5% CO_2 in air, the supernatants were harvested and assayed for IgG content using the enzyme-linked immunoabsorbance assay.

2.6.2 Lymphocyte proliferation

PBMC (10^6 cm^{-3}) were cultured in 0.2 cm^3 aliquots of RPMI 1640 containing 10% FCS in round bottomed microtitre plates. Cultures were set up in the absence or presence of PHA, $1 \mu\text{g cm}^{-3}$. The cells were incubated for 72 hours at 37C in a humid atmosphere of 5% CO_2 in air. 4 hours before termination of the incubation the cells were pulsed with $0.5 \mu\text{Ci}$, tritiated thymidine (specific activity 5 ci mmol^{-1}). The cells were harvested onto glass fibre discs and dried. These were then placed into vials containing 2 cm^3 scintillation fluid and the uptake of radioisotope determined by liquid scintillation spectrometry.

2.6.3 Enzyme-linked immunoabsorbance assay (ELISA) for IgG

0.2 cm^3 of an optimal dilution of a goat anti-human polyvalent immunoglobulin was dispensed into each ELISA cuvette well. The cuvettes were incubated in a humid box for 1 hour at 37C and subsequently overnight at 4C. They were washed extensively with PBS/Tween, the final wash being aspirated with a mechanical pump. 0.2 cm^3 of each doubling dilution of the control serum or test supernatants (in PBS/Tween) was added (in duplicate) to the cuvettes, which were subsequently incubated in a humid box at 37C for 1 hour. After incubation, the washing procedure with PBS/Tween was repeated. 0.2 cm^3 of an optimal dilution (in PBS/Tween) of

the phosphatase conjugated anti-human IgG was added to each cuvette and the plates incubated at 37C for 1 hour in a humid box. After extensive washing with PBS, 0.2 cm³ alkaline phosphatase substrate solution (p-nitrophenol phosphate) in ethanolamine buffer (1mg cm⁻³) was added to each cuvette. The colour was allowed to develop at room temperature and when the positive control serum reached an absorbance value of 2.0 at 405 nm, the reaction was stopped by the addition of 0.2 cm³ 1.5M NaOH. The optical density of each cuvette was then determined at 405nm. The level of IgG synthesis was calculated by reference to a calibration curve obtained from known amounts of IgG in the assay system.

2.6.4 Serum SH assay

Serum samples were stored at -20C before use. 0.05 cm³ of serum was diluted with 0.75 cm³ of PBS and reacted with 0.2 cm³ of freshly prepared 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) solution (2mM) for 5 minutes at 37C. The absorbance at 440 nm. were measured against a serum blank. This value was converted to a serum SH concentration by reference to a calibration curve obtained from known amounts of reduced glutathione in the assay system.

2.6.5 Cell surface SH assay

6,6' dithiodinicotinic acid (6,6'-D) blocks cell surface

SH groups but does not penetrate the cell (Mehrishi and Grassetti, 1969). The mixed disulphide formed between the cell thiols and 6,6'-D can be split by treatment with a thiol such as glutathione with the subsequent release of thione into the supernatant. This can be measured spectrophotometrically at 344 nm. and used to determine the number of SH groups on the cell surface.

20×10^6 PBMC in 0.2 cm^3 CMFSS were treated with 6,6'-D at 37C for 5 minutes. The cells were washed three times and resuspended in 0.2 cm^3 CMFSS. The treated cells were then allowed to react with 10^{-3} M. glutathione to release the thione, bound to the cell surface, into the supernatant. The cell suspensions were centrifuged and the supernatants collected. The amount of thione released was measured spectrophotometrically at 344 nm. The amount of surface SH groups present was calculated by reference to a calibration curve obtained from known amounts of reduced glutathione in the assay system.

2.6.6 Blockade of serum SH groups

Serum from healthy human controls was treated with a range of concentrations of iodoacetamide (IOA) for 1 hour at 37C to block the SH groups. Excess IOA was removed by dialysis against CMFSS overnight at 4C.

2.6.7 Blockade of cell membrane SH groups

Cell surface SH groups were blocked by preincubating the cells for 1 hour at 37C with an irreversible non-penetrating SH blocking agent, p-hydroxymercuriphenylsulphonate (PHMPSA) (Tsan and Berlin, 1971). The cells were then washed three times with CMFSS and resuspended in RPMI 1640.

2.6.8 IL-1 Production

Monocytes were separated from PBMC by centrifugation on 52% Percoll as previously described. Monocytes (10^6) were incubated in 1 cm³ of RPMI 1640 containing 10% FCS and 1 µg of LPS for 24 hours at 37C, in the presence or absence of 2-ME (5×10^{-5} M). The supernatants were then collected and stored at - 20C prior to IL-1 assay.

2.6.9 IL-1 Assay

3-6 week old C₃H/HeJ mouse thymus tissue were finely minced in DAB containing 2% FCS and dispersed by passage through a sterilised nylon screen. Large fragments were removed and discarded. Adherent cells were removed by passage through a Sephadex G10 column prewarmed at 37C. The cell suspension was washed three times in DAB/2% FCS at 4C. 0.1 cm³ aliquots of test supernatants were added in doubling dilutions to flat-bottomed microtitre plates. Cells (10^6 in

0.1 cm³ of RPMI 1640 containing 1% FCS, 5 x 10⁻⁵ M 2ME and 3 µg PHA) were incubated with tritiated thymidine (5 cpm mol⁻¹) at 37°C for 68 hours and then were pulsed for 4 hours. After harvesting the amount of thymidine uptake was used as an estimate of cell proliferation. The capacity of each of the test supernatants to induce cell proliferation was calculated using probit-analysis and the results plotted on an arbitrary scale of 1-100 units.

2.6.10 Production of IL-2

PMBC (10⁶ cm⁻³) were incubated in RPMI 1640 containing 10% FCS and 1 mg cm⁻³ PHA in a humidified atmosphere of 5% CO₂ in air at 37°C for 24 hours. At the end of the incubation period the cells were washed three times in RPMI 1640, resuspended and incubated similarly for another 24 h without PHA or FCS in 1 cm³ lymphocyte culture tubes. The tubes were then centrifuged at 2,000 rpm for 15 min and the supernatants collected. The supernatants were frozen at - 20°C prior to assay.

2.6.11 Assay for IL-2 activity

Normal human PBMC activated with PHA were maintained in culture for at least 2 weeks in RPMI 1640 supplemented every 4 days after the first week in culture with both FCS and lectin-free IL-2. The cells were used 4 days after the last addition

of IL-2 for the assay of IL-2 activity. They were washed in CMFSS and placed in microtitre plates (10^4 cells in 0.2 cm^3 RPMI 1640 with 10% FCS). The supernatants being tested were added at eight doubling dilutions. The plates were incubated at 37°C for 48 hours. Controls with resting cells were included and all assays were carried out in triplicate. IL-2 activity was measured by the ability of test supernatants to induce cell proliferation. The capacity of each of the test supernatants to induce cell proliferation was calculated using probit-analysis and the results plotted on an arbitrary scale of 0-100 units.

2.7 RAT CELL PREPARATION

PVG rats were injected in the base of the tail with 1 cm^3 of an emulsion containing 10 mg of ovalbumin (OVA) in Freund's complete adjuvant. After 11 days the rats were sacrificed. The lymph nodes were removed aseptically and minced in Dulbecco's A and B buffer containing 7% FCS (DAB/2% FCS) and dispersed by passage through a sterilised nylon screen. Large fragments were removed and discarded. Adherent cells were removed by passage through a Sephadex G10 column prewashed at 37°. The cell suspension was washed three times in DAB/2% FCS.

Spleens were removed from non-immunised PVG rats and the cells separated in a similar manner. Extraneous red cells

lysed with ammonium chloride buffer for 5 mins. and the cells were washed five times in DAB/2% FCS.

2.8 RAT CELL CULTURE

10^6 lymphnode cells and 10^6 spleen cells (macrophages) were cultured alone or in combination. The cells were cultured in RPMI 1640 containing 5% FCS and $10 \mu\text{g cm}^{-3}$ OVA in 0.2 cm^3 flat-bottomed microtitre plates for 72 hours. 4 hours before termination of the incubation the cells were pulsed with $0.5 \mu\text{Ci}$, tritiated thymidine (specific activity 5 Ci mmol^{-1}). The uptake of the radioisotope was determined by liquid scintillation spectrometry.

CHAPTER THREE

Results

3.1 SULPHYDRYL-DEPENDENT MONOCYTE-LYMPHOCYTE INTERACTIONS IN RHEUMATOID ARTHRITIS

3.1.1 The role of SH groups in the pathogenesis of Rheumatoid Arthritis

The role of SH groups in the pathogenesis of RA is poorly understood. This study was aimed at bringing together the experimental evidence of SH involvement in the immune response, the low serum SH levels in RA patients and the sulphhydryl-reactivity of antirheumatic drugs. The investigation assessed the role of SH groups in regulating monocyte-lymphocyte interactions in RA and the modulation of these parameters during treatment with D-pen.

3.1.2 The role of SH groups in the immune response

Serum SH levels are essentially determined by the number of albumin molecules which possess a free thiol. The thiol dependent serum factor (MaSF) described by Opitz et al. (1980) is biochemically very similar or identical to albumin. This may provide a link between the low serum SH level and immunological abnormalities characteristic of RA. If free SH

groups on albumin are necessary for a normal immune response in vivo, it may be prevented by depressed serum thiols. Serum albumin has been shown to be absolutely necessary for human lymphocyte proliferation in response to PHA (Polet and Spieker-Polet, 1975; Spieker-Polet and Polet, 1976; Arai et al., 1977). This provides a useful assay system for determining the effect of reducing the available number of free albumin thiols on the immune response. This may be achieved in two ways, the albumin concentration in the assay may be reduced or the serum SH may be blocked biochemically.

3.1.3 The effect of reducing the available free thiols on mitogen stimulated PBMC proliferation in vitro

Experiments were carried out to determine whether the SH group on the albumin molecule is necessary for lymphocyte activation. The optimum concentration of PHA, $1\mu\text{g cm}^{-3}$, was determined in preliminary experiments. The use of albumin in the culture system made it possible to reduce the SH level simply by reducing the albumin. The results of this experiment are shown in Fig. 4. Maximum proliferation was obtained with the addition of 4mg cm^{-3} BSA. This is a similar amount to that provided by the addition of 10% FCS. However, there was no significant difference between the responses with 4 and $1\mu\text{g cm}^{-3}$ BSA. A four-fold reduction in albumin concentration did not affect cell proliferation. In further

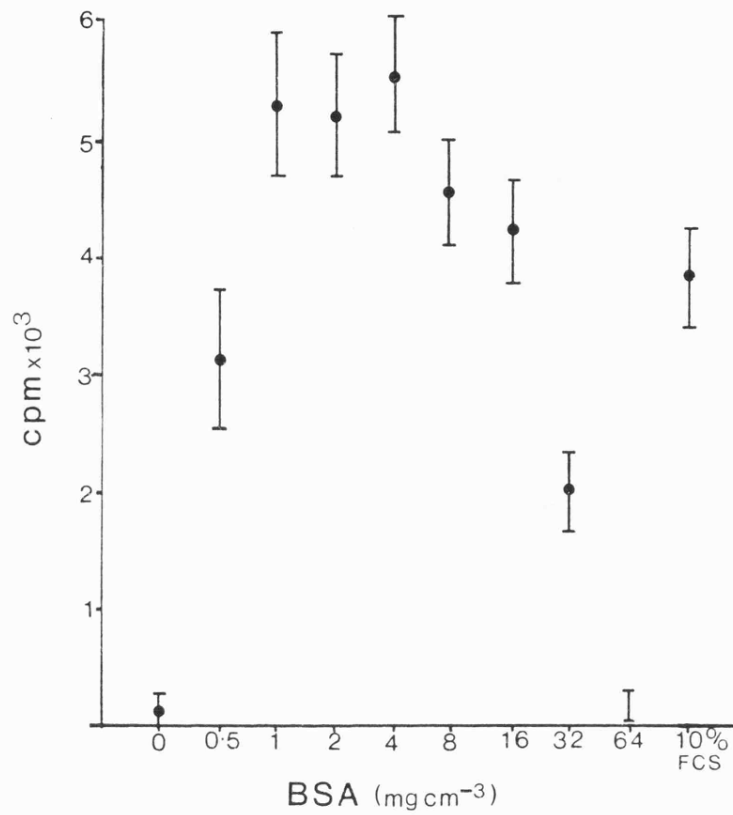


Fig. 4. Effect of BSA on PHA response in serum free culture medium. The bars represent % stimulation (mean \pm l.s.d.) compared to cells cultured in absence of BSA or FCS. ($n = 6$). Maximum proliferation was obtained in cultures containing 4 mg cm^{-3} BSA.

assays human serum albumin (HSA) was added to the culture in place of BSA (Fig. 5). Again maximum proliferation was obtained with $1 - 4 \text{ mg. cm}^{-3}$. These data suggest that serum albumin thiols are not rate limiting for the in vitro immune response.

Experiments were carried out to determine whether or not a thiol dependent serum factor other than albumin is involved in the human immune response. Whole human serum was separated into fractions by passage through a Sepharose 6B column. The fractionation was monitored using a spectrophotometer. Five separate peaks were observed and these are shown in Fig. 6. The fractions representing each peak were pooled and concentrated to their original volume using an Amicon filter. The ability of each of the fractions to promote PBMC proliferation in the PHA assay was determined. The results of this experiment are shown in Fig. 7. The only fraction to promote PBMC proliferation was fraction 4. This is the fraction which contains albumin molecule - molecular weight 68,000. This finding does not however rule out the possibility of the existence of a thiol dependent serum factor, which is similar, but not identical, to albumin. This was investigated by reducing the SH level of whole human serum.

Normal serum was incubated with an irreversible SH-blocking agent (IOA) to produce a range of serum SH levels,

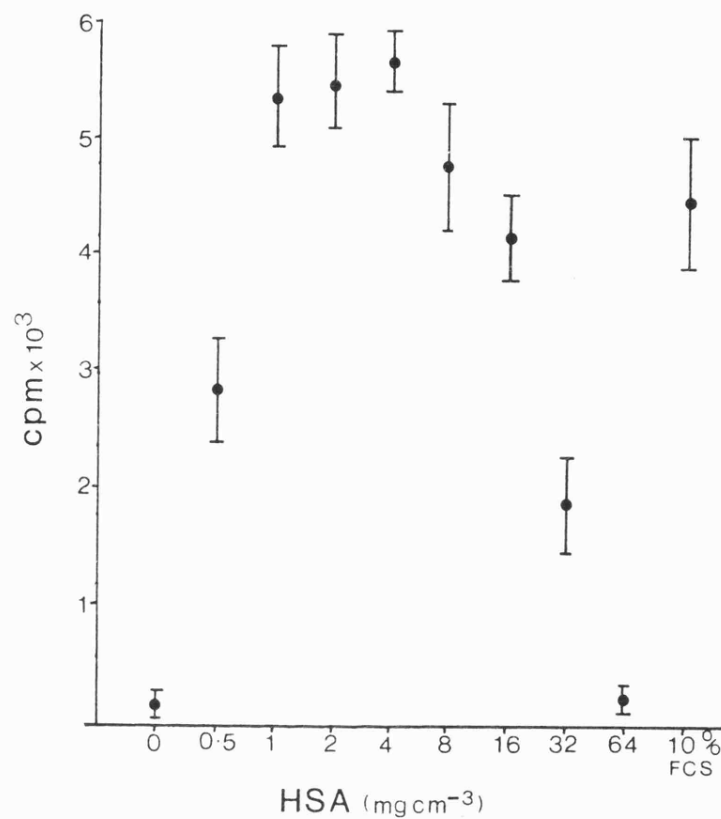


Fig. 5. Effect of HSA on PHA response in serum free media. The bars represent % stimulation (mean \pm l.s.d.) compared to cells cultured in the absence of HSA or FCS $n = 4$. Maximum proliferation was obtained in cultures containing 4 mg cm^{-3} HSA.

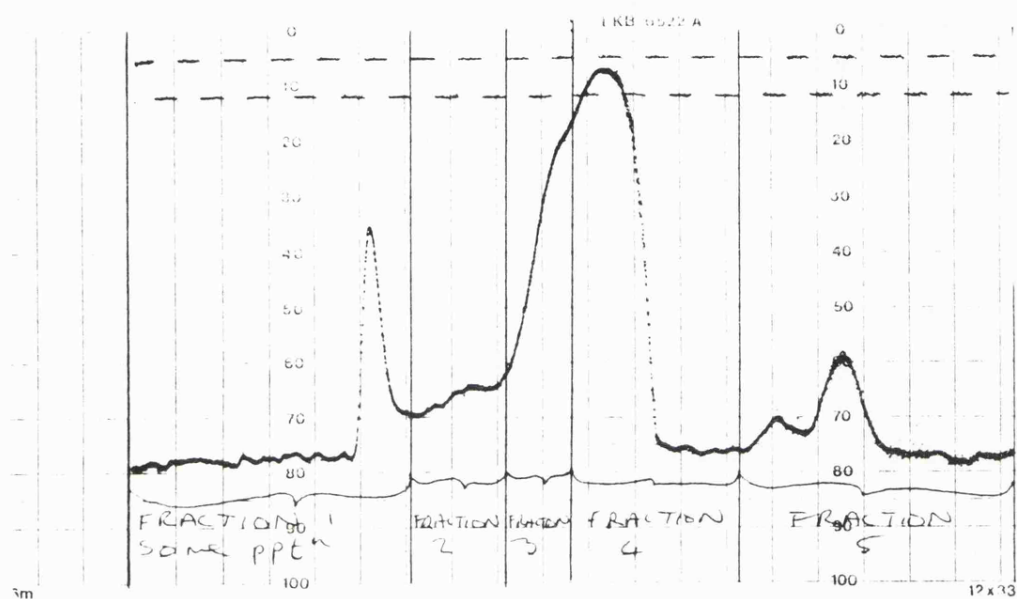


Fig. 6. Protein fractions separated from whole human serum by Sepharose 6B chromatography.

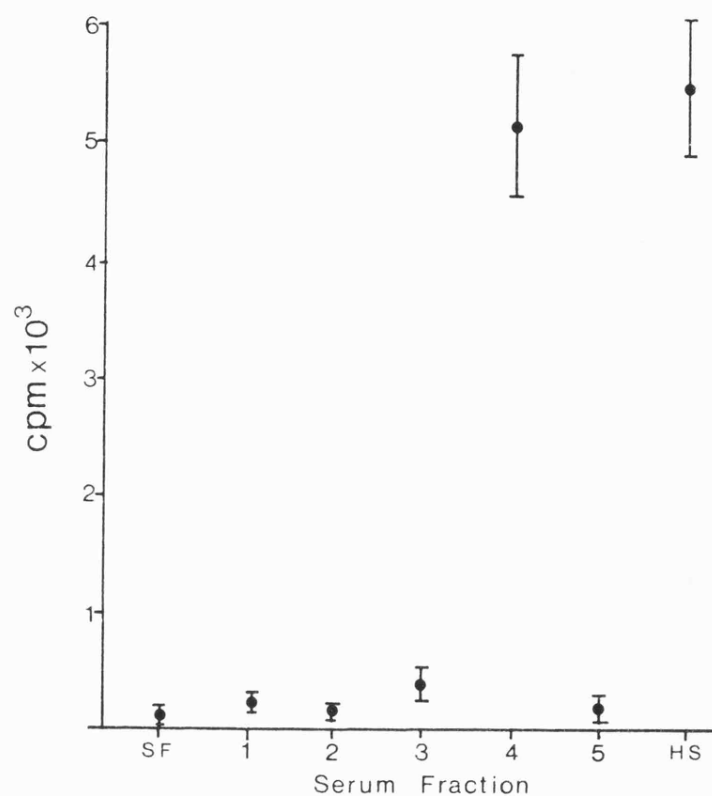


Fig. 7. PHA-stimulated PBMC proliferation in response to each of five human serum protein fractions. Proliferation occurs only in the presence of fraction 4.

100 - 600 $\mu\text{M dm}^{-3}$ (Fig. 8). The serum was then added to autologous PBMC in a culture system containing PHA.

The results of these experiments are shown in Fig. 9. This shows that blockade of serum SH groups has no significant effect on PHA lymphocyte proliferation in vitro in the serum SH range representative of either normal healthy individuals, (600 $\mu\text{M dm}^{-3}$ to 400 $\mu\text{M dm}^{-3}$) or for patients suffering from severe RA, (250 $\mu\text{M dm}^{-3}$ to 200 $\mu\text{M dm}^{-3}$). Any SH-dependent serum factor does not appear to be rate limiting.

3.1.4 The effect of cell surface SH blockade on PHA-stimulated PBMC proliferation in vitro

It has been demonstrated that the addition of SH blocking agents to cell cultures results in the inhibition of lectin-induced proliferation (Chaplin and Wedner, 1978). Experiments were carried out to determine whether this inhibition was due to cell surface blockade rather than an effect on serum.

The effect of the presence of a non-penetrating SH blocking agent PHMPA on healthy PBMC cultures is shown in Fig. 10. PHMPA inhibited lymphocyte proliferation in a dose-dependent manner. 50% inhibition was achieved by the addition of 10 μM PHMPA and proliferation was completely abolished in the presence of 100 μM PHMPA.

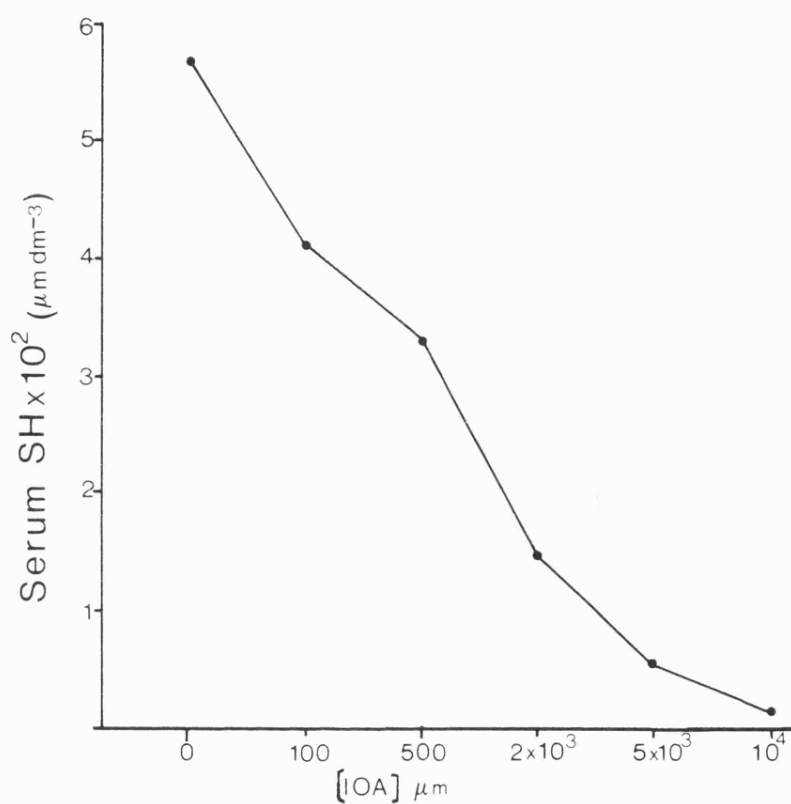


Fig. 8. The effect of incubating serum with IOA on free SH levels. Incubation with a range of 500 - 10,000 μM IOA produces serum SH levels of between 600 - 100 $\mu\text{m dm}^{-3}$.

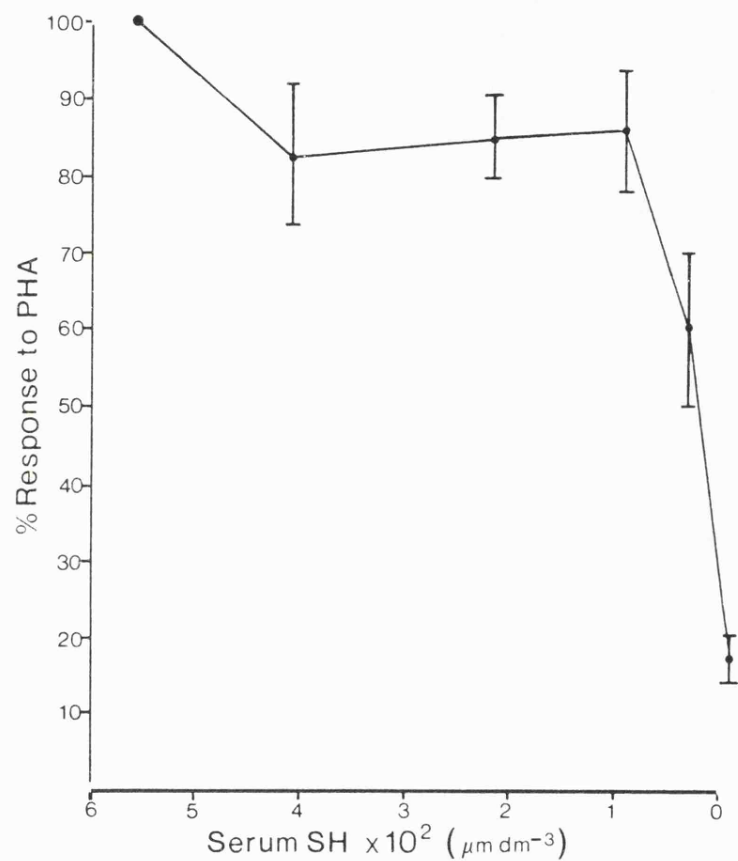


Fig. 9. Effect of serum blockade on PHA response ($n = 10$). The bars represent % response of lymphocytes cultured with untreated serum (\pm l.s.d.). Serum levels in the range 600 - 100 μM did not significantly affect PHA stimulated lymphocyte proliferation ($p > 0.05$, Mann-Whitney U test).

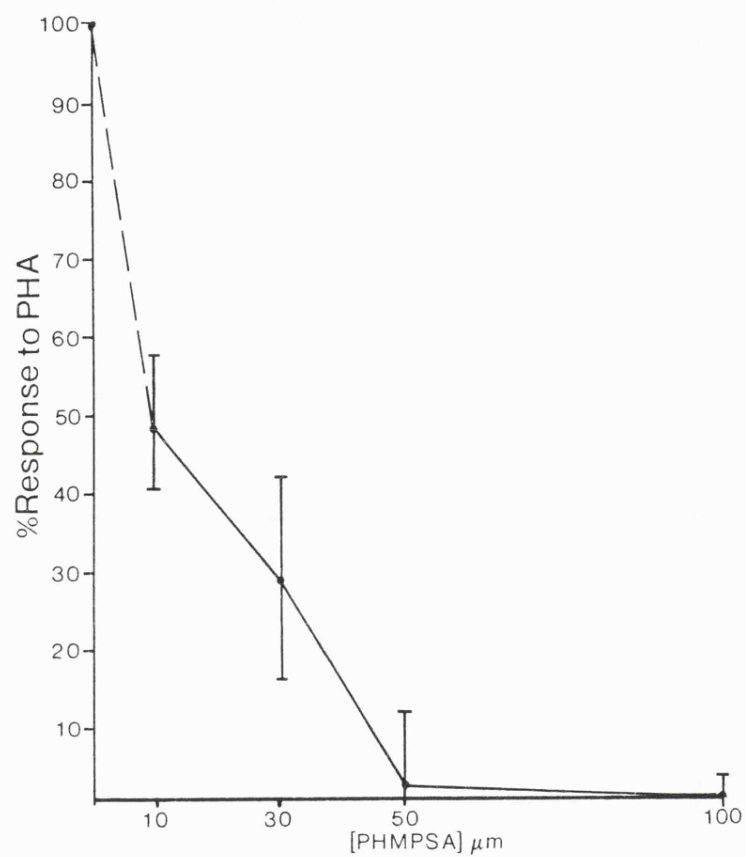


Fig. 10. Effect of the addition of PHMPSA on PHA stimulated lymphocytes ($n = 7$). Bars represent % response to PHA compared to cells not treated with PHMPSA (± 1 s.d.) The ID₅₀ of PHMPSA was found to be 10 μM .

The use of a non-penetrating SH blocking agent ensures that the inhibition is not due to intra-cellular SH blockade but it does not rule out the possibility that the effect is due to SH blockade of another component of the culture medium. To determine whether the inhibition was due to blocking cell surface SH group the PBMC were pre-incubated with PHMPSA and then washed prior to culture.

Pre-incubation of the PBMC resulted in similar dose-dependent inhibition of cell proliferation (Fig. 11). The effect of PHMPSA, was therefore, exerted on cell surface SH groups, and not on a component of the culture medium. Treatment of PBMC with PHMPSA did not affect cell viability as assessed by trypan blue exclusion after the 7-day culture period at 37C.

3.1.5 The role of thiols in monocyte accessory cell functions in the immune response in vitro.

The above data indicate that free SH groups on the PBMC surface are necessary for proliferation. The reason for their necessity was then investigated. Other investigators have shown that in animal culture systems thiols are involved in the accessory role of macrophages. Experiments were carried out to determine if this was so in the human immune response. The PBMC were separated into lymphocytes and monocytes by density flotation on 52% Percoll. The monocytes and

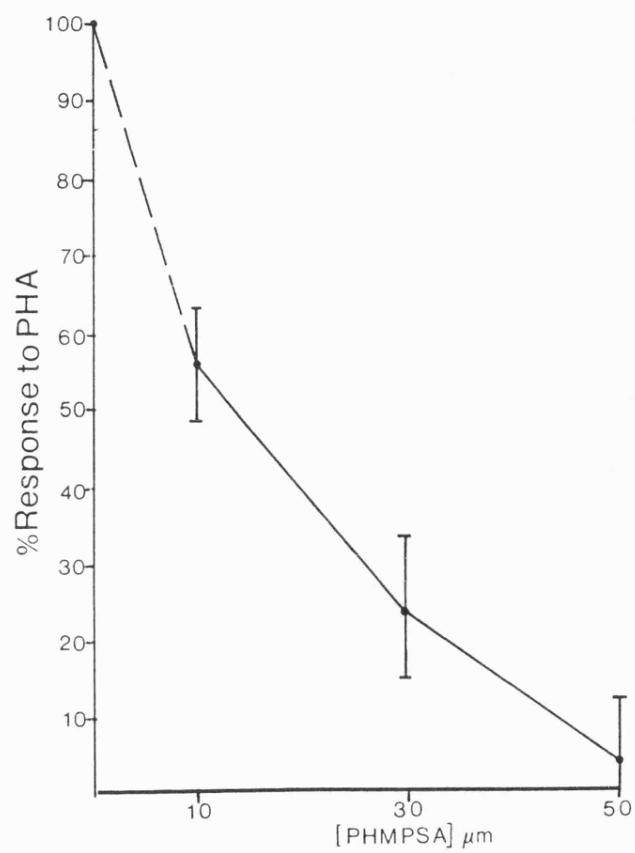


Fig. 11. Effect of preincubation of PBMC with PHMPSA on PHA stimulated cell proliferation ($n = 5$). Bars represent % response to PHA compared to cells not preincubated with PHMPSA (± 1 s.d.). The ID_{50} is again $10 \mu\text{M}$.

lymphocytes were then recombined. In the cell proliferation assay it proved impossible to consistently remove sufficient monocytes to abolish proliferation in response to PHA. Similar experiments were carried out using a PWM stimulated IgG synthesis assay system to determine whether this would provide a more consistent assay for the study of monocyte lymphocyte interactions.

Initial experiments showed that a final dilution of 1/1000 PWM was optimal in this assay - although approximately 10% of those subjects studied did not respond to PWM (Fig. 12). IgG production was measured using an ELISA technique. The optical density was measured and the level of IgG was calculated by reference to a calibration curve - an example of which is illustrated in Fig. 13.

Pre-incubation of PBMC with PHMPA also resulted in inhibition of PWM-stimulated IgG synthesis (Fig. 14). PBMC from healthy controls produced $2,234 \pm 173$ ng IgG 10^{-6} cells in the presence of PWM. Only small amounts of IgG were generated in the absence of PWM (59 ± 20 ng 10^{-6} cells). Pre-incubation of the cells for 60 mins. with 10 μ M PHMPA resulted in IgG synthesis being reduced to 1023 ± 172 ng IgG 10^{-6} PBMC (approximately 50% inhibition). Again, 50 μ M PHMPA abolished IgG synthesis (71 ± 60 ng 10^{-6} cells).

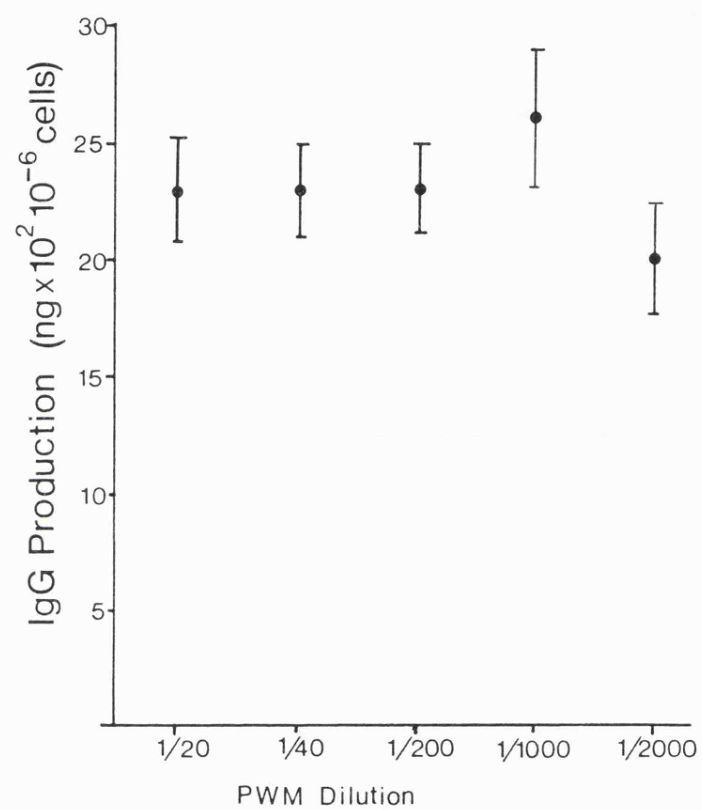


Fig. 12. Optimum PWM dilution required for IgG synthesis by PBMC. Optimal dilution PWM = 1/1000.

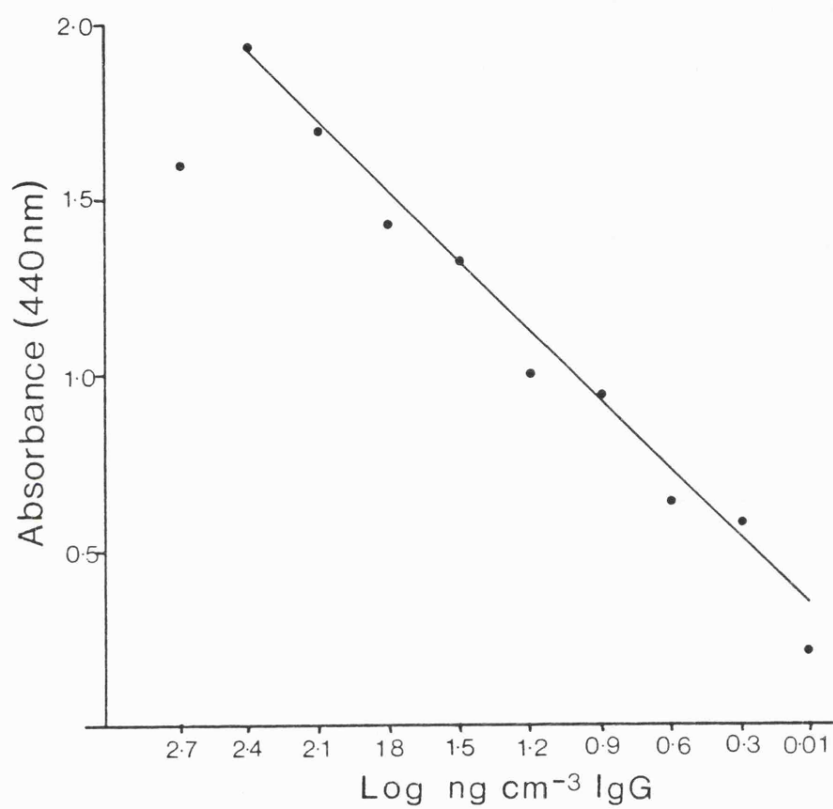


Fig. 13. IgG calibration curve. The level of IgG production by PWM stimulated cultures was determined by reference to a calibration curve obtained from known levels of IgG.

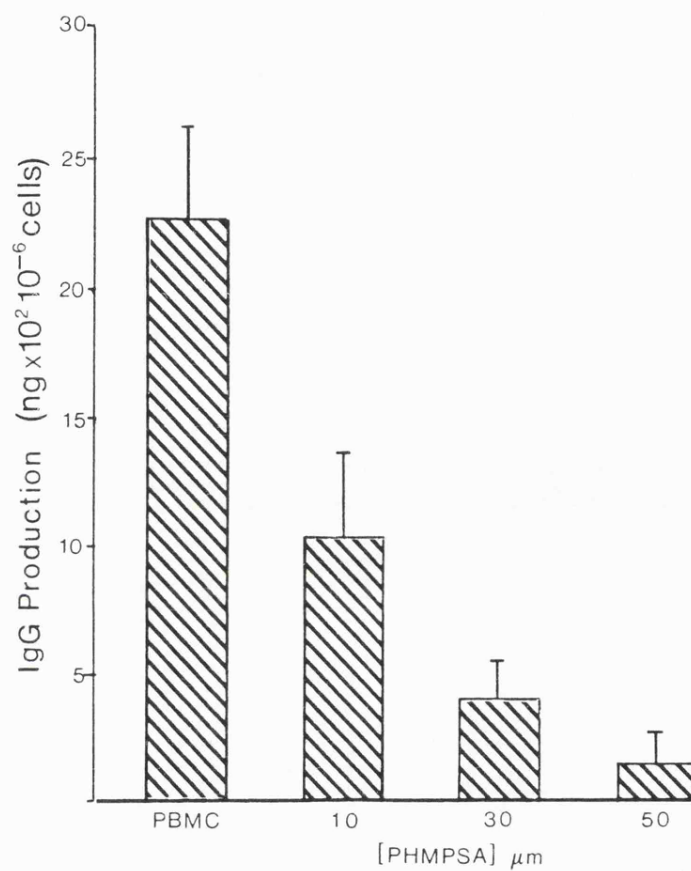


Fig. 14. Effect of preincubation with PHMPSA IgG production ($n = 3$). Each histogram represents the mean IgG production in $\text{ng} \times 10^2$ 10^{-6} cells ($\pm 1.\text{s.d.}$). ID_{50} n. of PHMPSA was found to be $10 \mu\text{M}$.

The synthesis of IgG in response to PWM was more easily inhibited by monocyte depletion on 52% Percoll but this was not always achieved. The addition of an adherence step by which any residual monocytes in the lymphocyte fraction were removed by passage through a Sephadex G10 column proved very consistent and provided a reliable assay for the study of monocyte-lymphocyte interactions.

Evidence supporting monocyte dependence of IgG production by PWM stimulated PBMC and the involvement of cell surface SH groups in this accessory cell function is presented in Fig. 15. It is clear that purified lymphocytes cannot respond to PWM in the absence of monocytes. Recombination of lymphocytes and monocytes (ratio 5:1) gave a full recovery of IgG synthesis. However, if either the monocytes or the lymphocytes were pre-incubated with PHMPSA, IgG synthesis was abolished. Treatment of the monocytes or lymphocytes with PHMPSA showed a dose dependent inhibition similar to that seen by PWM stimulated IgG synthesis by PBMC and PHA stimulated lymphocyte proliferation (Fig. 16).

Cell surface SH groups are, therefore, necessary for some form of interaction between lymphocytes and monocytes and their blockade results in an abnormal immune response in vitro.

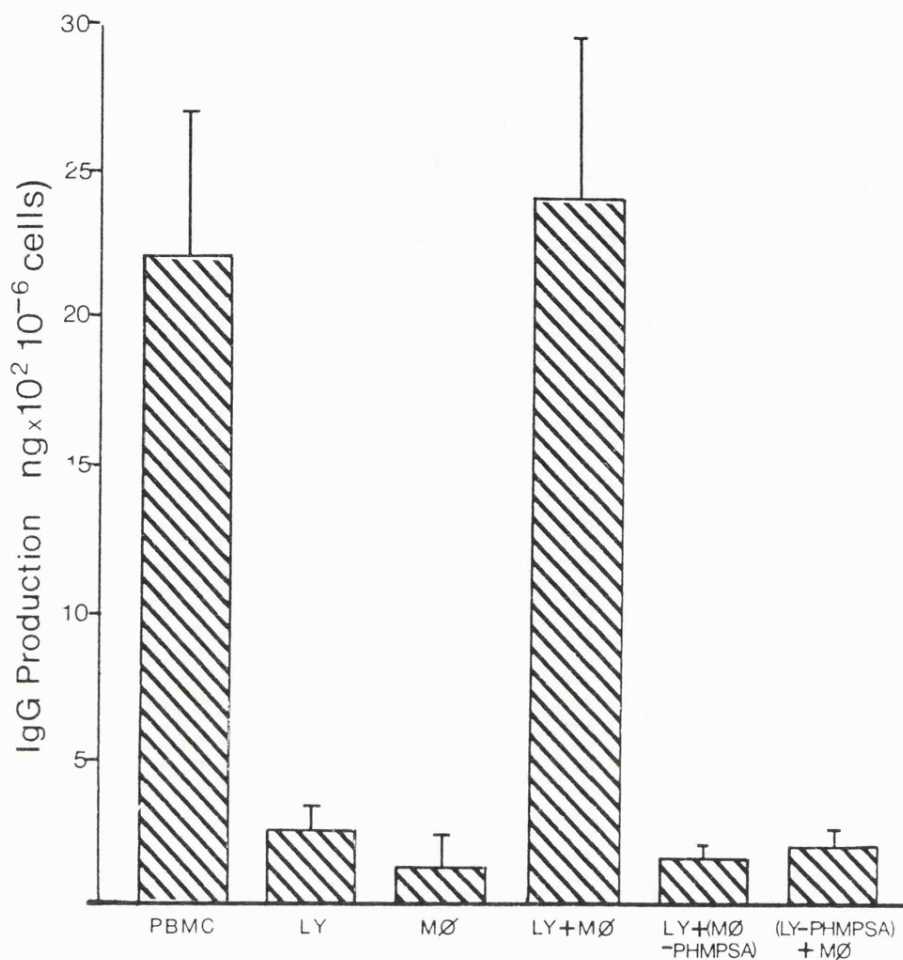


Fig. 15. IgG production by healthy control mononuclear cell fractions stimulated with PWM ($n = 6$). Each histogram represents the mean IgG production ($\text{ng} \times 10^2 10^{-6} \text{ cells}$) ($\pm 1.s.d.$) Cell fractions tested were: PBMC = peripheral blood mononuclear cells; Ly = purified lymphocytes; MØ = enriched monocytes; Ly + MØ = reconstituted mixture of lymphocytes and monocytes, ratio 5:1; Ly + MØ - PHMPSA) = reconstituted mixture of untreated lymphocytes and monocytes (pre-treated with the surface SH blocking agent PHMPSA) ratio 5:1, Ly-PHMPSA + MØ = reconstituted mixture of monocytes and lymphocytes pretreated with PHMPSA.

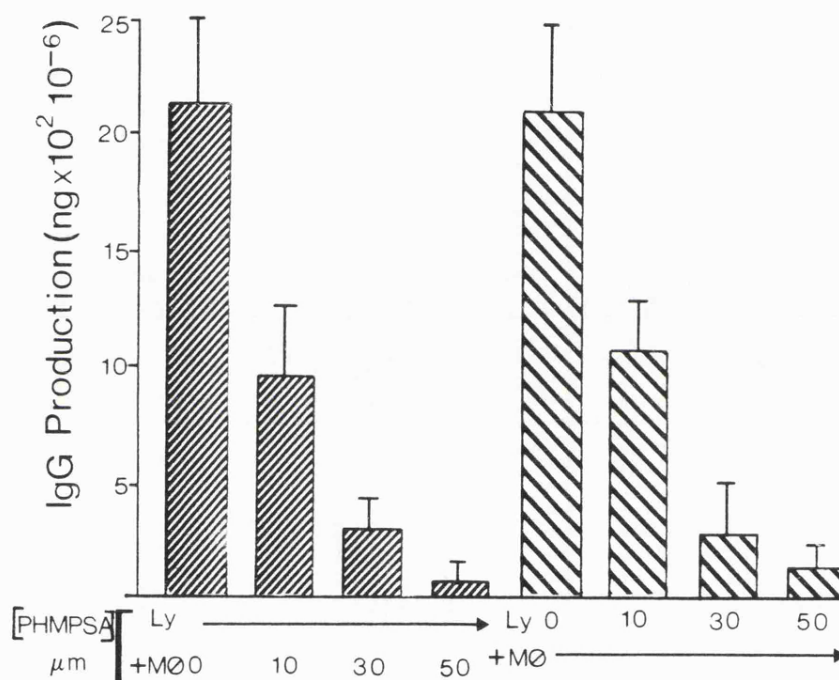


Fig. 16. Effect of preincubating either lymphocytes or monocytes separately with PHMPSA ($n = 5$). The histograms on the left hand side each represent IgG production ($\text{ng} \times 10^2 10^{-6}$ cells) by lymphocyte and PHMPSA preincubated monocytes (Ratio 5:1). The histograms on the right represent IgG production ($\text{ng} \times 10^2 10^{-6}$ cells) by lymphocytes pretreated with PHMPSA and untreated monocytes (Ratio 5:1), (± 1 s.d.) Inhibition of IgG synthesis is dose dependent whether the lymphocytes or the monocytes are treated.

This may explain the abnormal immune response characteristic of RA. If blocked SH groups extend from the serum to the PBMC surface it may result in an aberration of the immune system.

3.1.6 Experiment to determine if cell surface SH groups correlate with serum SH levels

Experiments were therefore carried out to determine if the cell surface SH groups correlated with serum levels. Cell surface SH may be measured by incubating the cells with 6,6'-D. This compound reacts with the cell surface thiols. After excess 6,6'-D has been removed part of the 6,6'-D molecule attached to the cell surface can be cleaved off. This compound, a thione, can be measured spectrophotometrically at 344 nm. and is proportional to the number of free cell surface SH groups (Mehrishi and Grassetti, 1969). In order to demonstrate that this assay was a true indication of the number of free surface thiols PBMC were obtained from healthy volunteers and the cell surface thiols blocked with PHMPA prior to assay. The PBMC were then incubated with 6,6'-D. The results presented in Fig. 17 show that the amount of thione released parallels the thiol blockade by PHMPA.

Serum and PBMC were obtained from healthy volunteers and rheumatoid patients in order to provide a range of serum SH levels. The cell surface SH groups and serum SH levels were

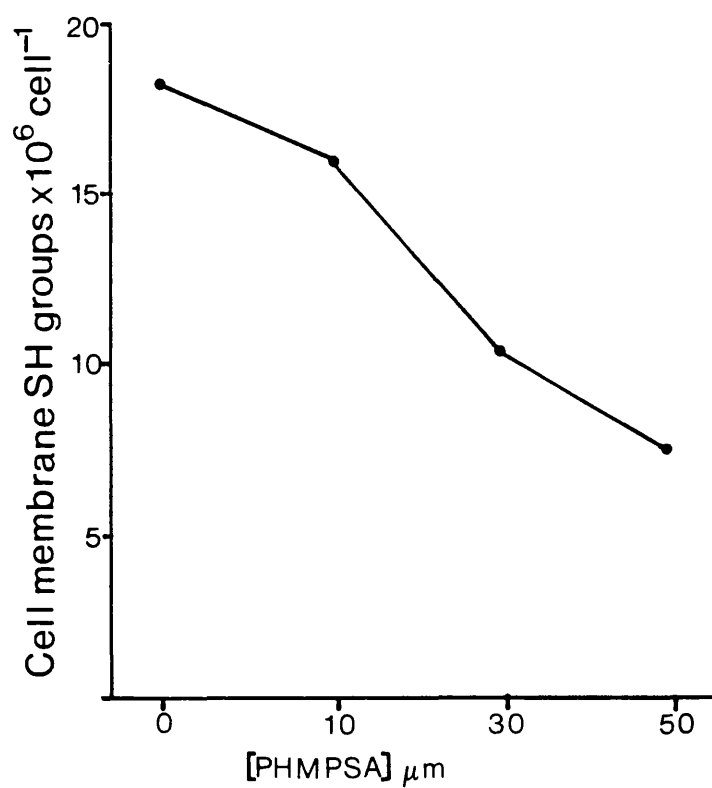


Fig. 17. Measurement of cell surface thiols after pre-incubation with PHMPSA. Cell surface thiols are reduced in a dose dependent manner after preincubation with PHMPSA.

measured. The results are shown in Fig. 18. Serum and cell surface SH groups do have an association but do not correlate ($r = 0.615$, $p > 0.05$).

3.1.7 The effect of thiol compounds on IgG synthesis by PBMC from rheumatoid patients

If cell surface thiols on PBMC are blocked in rheumatoid patients resulting in immunological abnormalities, the addition of a reducing agent may restore the normal immune response. This was studied in vitro using the IgG assay. The effect of 2-ME on PWM stimulated IgG synthesis by unfractionated PBMC was determined. Initial experiments showed that the optimal level of 2-ME was $5 \times 10^{-5} \text{M}$ (Fig. 19). PBMC from healthy volunteers produced $2345 \pm 554 \text{ ng IgG } 10^{-6} \text{ cells}$ in the presence of PWM PBMC from patients with active RA (RA-NSAID) synthesised significantly less IgG than normal cells in this assay ($1623 \pm 450 \text{ ng } 10^{-6} \text{ cells}$, $P < 0.005$), whereas IgG production by PBMCs from D-pen treated patients (RA-D-pen) did not differ significantly from that of normal cells ($P > 0.05$) (Fig. 20). Fig. 20 also shows that the depressed level of IgG synthesis by RA-NSAID cells was enhanced to normal values by the addition of 2-ME ($5 \times 10^{-5} \text{ M}$) to the culture medium. This procedure did not significantly enhance IgG production by healthy or RA D-pen PBMC.

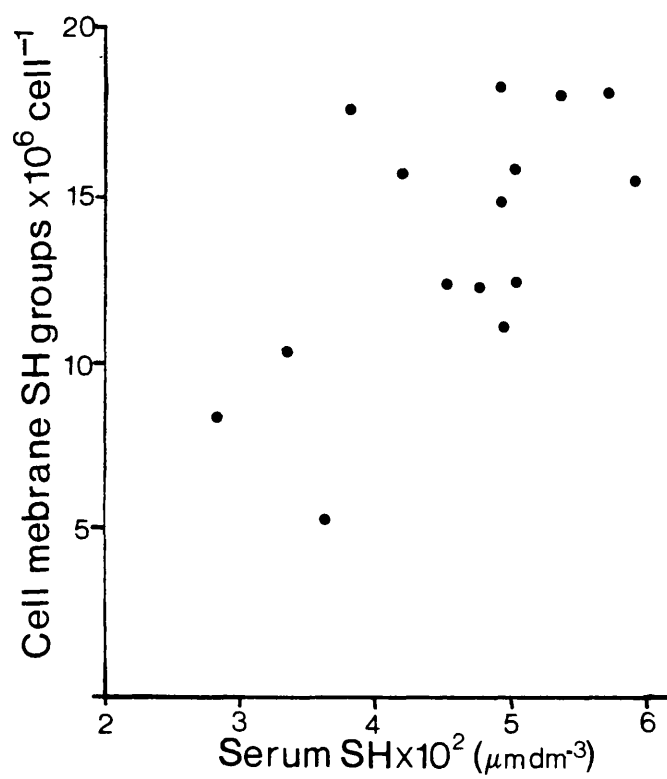


Fig. 18. Correlation of serum SH levels and cell surface SH groups ($r = 0.615$, $P > 0.05$).

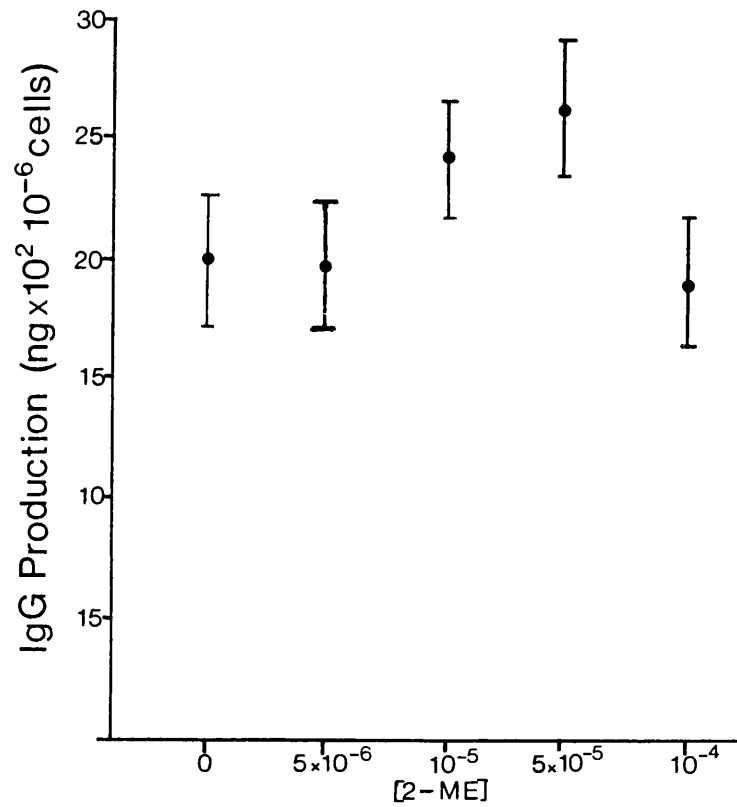


Fig. 19. Optimum level of 2-ME required to enhance IgG synthesis. The optimum level of 2-ME needed to enhance IgG synthesis was 5×10^{-5} M.

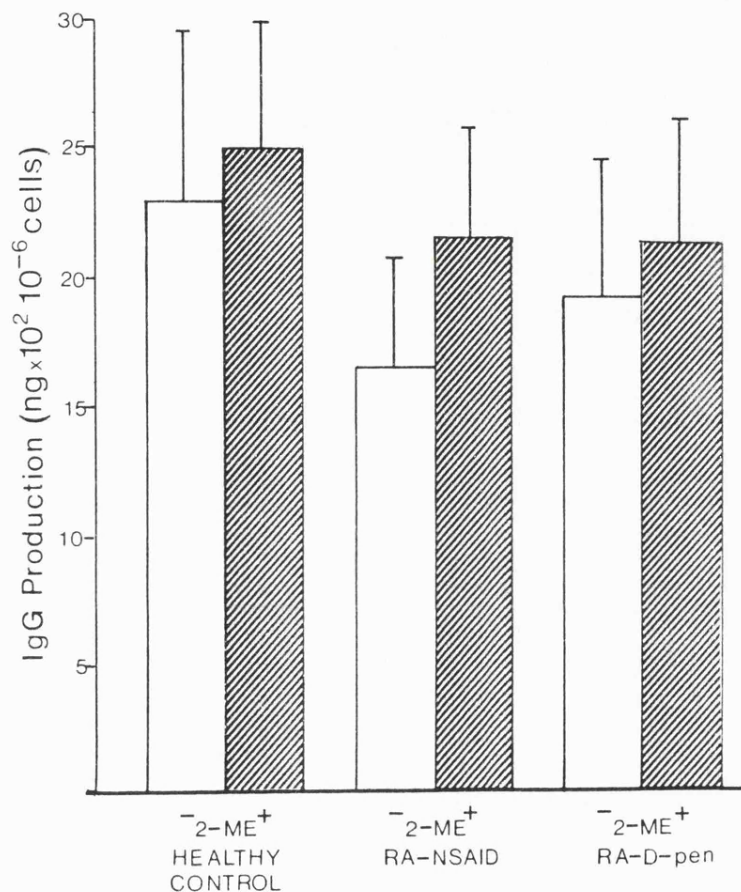


Fig. 20. Results are expressed as the mean IgG production (\pm l.s.d.) by 10^6 PBMC optimally stimulated with PWM. IgG production in unstimulated cultures was the same (66 ± 37 ng 10^{-6} PBMC) in all three groups. In the absence of 2-ME, PWM stimulated RA-NSAID cells generated less IgG than healthy cells ($P < 0.005$). IgG production by RA-D-pen cells did not differ significantly from that of healthy control cells ($P > 0.05$). The addition of 2-ME (5×10^{-5} M) to these cultures significantly increased IgG production by the RA-NSAID cells ($P < 0.005$) but not by healthy or RA-D-pen cells.

These data suggest that blocked SH groups on the PBMC surface do result in depressed IgG synthesis by rheumatoid cells. Reduction with 2-ME resulted in normal IgG production. Patients treated with D-pen also showed a normal response which may suggest that this drug has a similar effect on PBMC to that of 2-ME.

3.1.8 The role of SH group monocyte-lymphocyte interaction in rheumatoid patients

The sulphydryl blocking data showed that blockade of either lymphocytes or monocytes resulted in inhibiting the immune response in vitro.

Experiments were carried out to determine whether both the rheumatoid monocytes and lymphocytes have to be treated with 2-ME to obtain enhanced IgG synthesis. Lymphocytes and monocytes were separated and half of each cell type pre-incubated with 2-ME. The cells were recombined in culture. The results presented in Fig. 21 show that both monocytes and lymphocytes from RA-NSAID patients have to be pretreated with 2-ME to achieve enhanced IgG synthesis. This together with the other data suggest that free cell surface thiols are involved in a physical interaction between the monocytes and the lymphocytes.

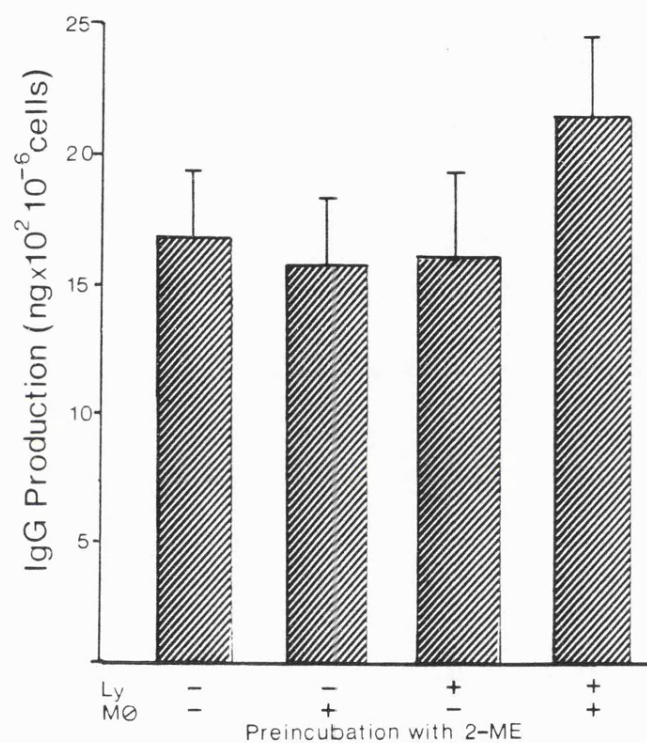


Fig. 21. The effect of pre-incubation of monocytes and lymphocytes from RA-NSAID patients. Both monocytes and lymphocytes have to be pre-treated with 2-ME to achieve enhanced IgG synthesis.

3.1.9 Experiments to determine whether blocked surface thiols affect monocyte accessory function in rheumatoid patients

These data suggest that the inhibition was due to an interaction between the lymphocytes and monocytes which may be relevant in RA.

The total dependence of PWM stimulated IgG production on the presence of monocytes permits a comparison of accessory cell function between individuals or patient groups, based on the titration curve of these cells added back to lymphocyte cultures. Fig. 22 illustrates the calculation of the monocyte ED₅₀, i.e. the number of monocytes that are needed to reconstitute 50% of the maximum IgG production by 10^6 lymphocytes. The mean titration curve for three groups, healthy controls, RA-NSAID and RA D-pen are shown in Figs. 23, 24 and 25 respectively. The addition of 2-ME to the pure lymphocyte culture system did not promote IgG production. Fig. 26 shows that medium monocyte ED₅₀ for healthy cells was found to be 400×10^{-6} lymphocytes. Accessory cell function did not change significantly in the presence of 2-ME, an expected finding in line with the data in Fig. 20. Monocytes from RA-NSAID patients showed impaired accessory function, with a median ED₅₀ value of 8000×10^{-6} lymphocytes. This differs significantly from both healthy cells ($P < 0.01$, Mann-Whitney U test) and RA D-pen monocytes ($P < 0.05$, Mann-Whitney U test). Accessory function of the latter, (median ED₅₀ 2,000

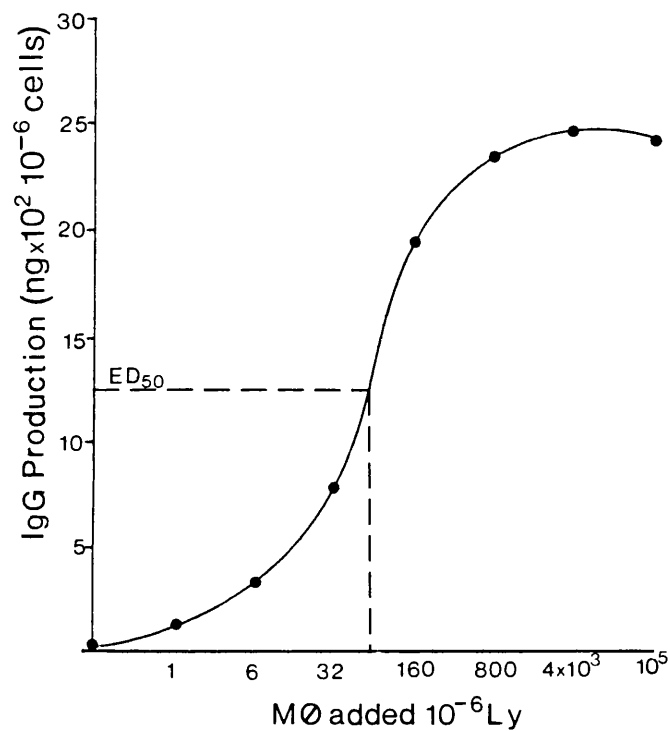


Fig. 22. Titration curve to illustrate calculation of monocyte ED_{50} . Standard deviation bars have been omitted for clarity. The dotted line illustrates the method of estimating the monocyte ED_{50} value, the number of monocytes 10^{-6} lymphocytes needed to promote half maximal IgG production.

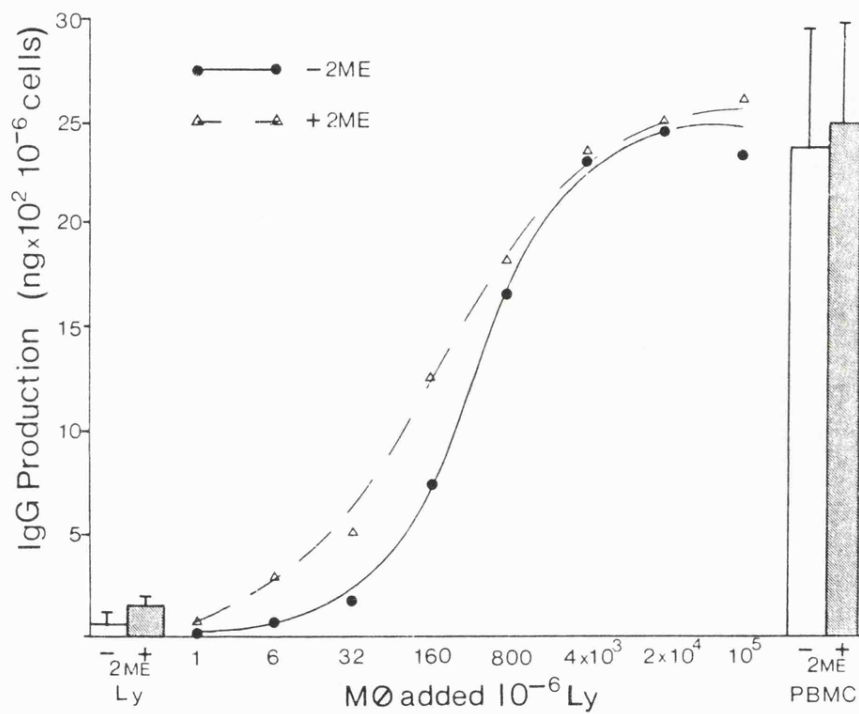


Fig. 23. Titration curve for healthy control monocyte accessory function in PWM stimulated IgG production. The histograms on the left show that 2-ME has no effect in the absence of monocytes. The histograms on the right show IgG synthesis (ng $\times 10^2$ 10^6 cells), by unfractionated PBMC cells. The graph gives the mean response for 12 experiments. Standard deviation bars have been omitted for clarity. The median monocyte ED_{50} is 400×10^{-6} lymphocytes.

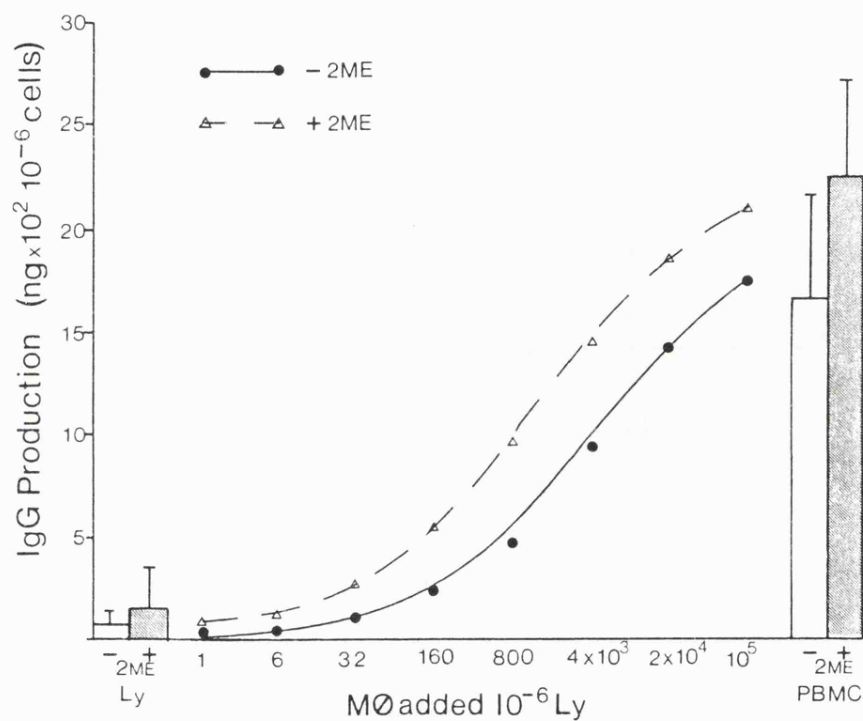


Fig. 24. Titration curve for RA-NSAID monocyte accessory cell function in PWM stimulated IgG production. The graph gives the mean response for 13 patients. The median monocyte ED_{50} is 8000×10^{-6} lymphocytes. The histograms on the right show IgG synthesis (ng $\times 10^2 \times 10^{-6}$ cells) by unfractionated cells.

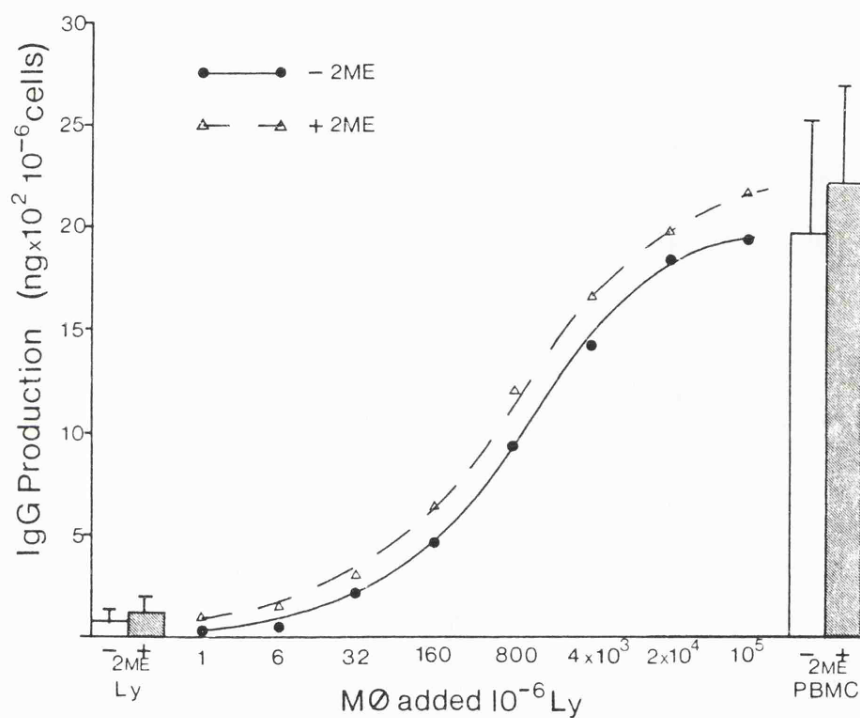


Fig. 25. Titration curve for RA-D-pen monocyte accessory cell function in PWM stimulated IgG production. The graph gives the mean response for 14 patients. The median monocyte ED_{50} is 2000×10^{-6} lymphocytes. The histograms on the right show IgG synthesis (ng $\times 10^2$ 10^{-6} cells) by unfractionated cells.

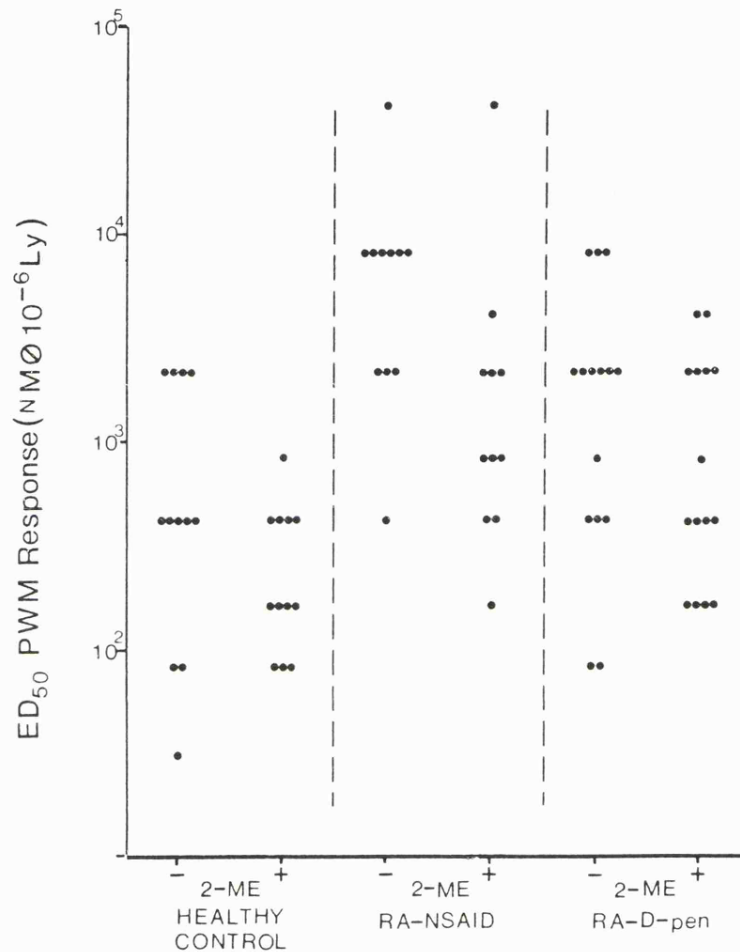


Fig. 26. Monocyte accessory function in PWM stimulated IgG production for the three patient groups. Cells were cultured without (-) or with (+) 2-ME ($5 \times 10^{-5}M$). Accessory function is presented as monocyte ED_{50} values estimated as illustrated in Fig. 22. In the absence of 2-ME, the RA-NSAID group differs significantly from the healthy controls ($P < 0.01$, Mann-Whitney U-test) and from RA-D-pen patients ($P < 0.05$). Data from the control and RA-D-pen groups are not significantly different. The addition of 2-ME enhances monocyte accessory function only in the RA-NSAID group ($P < 0.05$).

10^{-6} lymphocytes), was not significantly different from healthy controls. In the presence of 2-ME the accessory function of RA-NSAID monocytes was significantly enhanced ($P < 0.05$) but that of RA D-pen cells was not affected.

3.1.10 Serial study to determine the effect of D-pen therapy on monocyte accessory function in rheumatoid patients

Because the above patient groups were chosen specifically because they either had active RA or were established on D-pen with quiescent disease, a serial study was performed to determine whether the effects on monocyte accessory function were due to drug treatment or to disease activity. Initially 15 patients with active RA starting a course of D-pen therapy were studied. Due to early drop-out or inability of their cells to be stimulated by PWM, complete data over a 6 month period were obtained on only 8 patients. Three case reports are presented here and are representative of the patients studied. Unfortunately only one patient responded well to D-pen therapy (Case 1) and it must be noted that this patient was not suffering from severe disease. The clinical and laboratory assessment of these three patients are presented in Tables 1, 2 and 3. The clinical assessments were undertaken by Dr. A. Woolf, Senior Registrar at the Royal National Hospital for Rheumatic Diseases, Bath.

CASE 1

This was a 61 year old man who had a 4 year history of sero-positive RA. Extra-articular features were not present. He had not previously received any disease modifying therapy. This patient responded well to D-pen within 2-3 months with little active synovitis after 6 months therapy.

CASE 2

This was a 43 year old man with a 22 year old history of sero-positive RA, with no extra-articular features of the disease. This patient had received gold therapy in 1972 which resulted in a severe skin reaction. His symptoms improved after 3 months D-pen therapy with few symptoms after 6 months but active synovitis was still present on examination.

CASE 3

This was a 68 year old woman with a 9 year old history of sero-positive RA. Nodules and vasculitis were not present but the patient did suffer from Sjögren's syndrome.

This woman commenced D-pen therapy as an inpatient during which period she improved. Over a 5 month period the patient reported a non-specific improvement but still had widespread active synovitis on examination. No response was demonstrated

| PERIOD OF ASSESSMENT (MONTH) | 0 | 1 | 2 | 3 | 6 |
|--|-----------|-----------|-----------|-----------|-----------|
| D-pen Dose (mg day ⁻¹) | 0 | 125 | 250 | 375 | 375 |
| RITCHIE ARTICULAR INDEX | 8 | 8 | 7 | 7 | 3 |
| MEAN GRIP STRENGTH R | 300+/300+ | 300+/300+ | 300+/300+ | 300+/300+ | 300+/300+ |
| L | 300+/300+ | 300+/300+ | 300+/300+ | 300+/300+ | 300+/300+ |
| PLASMA VISCOSITY (cp) | 1.72 | 1.79 | 1.62 | - | 1.60 |
| C-REACTIVE PROTEIN (g dm ⁻¹) | - | 0.050 | 0.020 | <0.010 | <0.010 |
| SERUM SULPHYDRYL LEVEL $\mu\text{M dm}^{-3}$ | 396 | 368 | 406 | 418 | 477 |

TABLE 1. Clinical and laboratory assessment of patient who responded to D-pen therapy (Case 1).

| PERIOD OF ASSESSMENT (MONTH) | 0 | 1 | 2 | 3 | 6 |
|--|----------|------|---------|----------|-----------|
| D-pen Dose (mg day ⁻¹) | 0 | 125 | 250 | 375 | 250 |
| RITCHIE ARTICULAR INDEX | 8 | - | 4 | 4 | 3 |
| MEAN GRIP STRENGTH R | 250/290 | | 250/245 | 300/300 | 300+/300+ |
| L | 300/300+ | | 290/280 | 300/295+ | 300/300 |
| PLASMA VISCOSITY (cp) | 2.08 | 2.10 | 2.00 | 1.98 | 1.97 |
| C-REACTIVE PROTEIN (g dm ⁻¹) | - | - | 0.07 | 0.04 | 0.02 |
| SERUM SULPHYDRYL LEVEL $\mu\text{M dm}^{-3}$ | 240 | 197 | 302 | 328 | 317 |

TABLE 2. Clinical and laboratory assessment of patient who responded slowly to D-pen therapy (Case 2).

| PERIOD OF ASSESSMENT (MONTH) | 0 | 1 | 2 | 3 | 6 |
|--|------|-------|-------|-------|-------|
| D-pen Dose (mg day ⁻¹) | 0 | 125 | 250 | 375 | 0 |
| RITCHIE ARTICULAR INDEX | 44 | 25 | 26 | 37 | 37 |
| MEAN GRIP STRENGTH R | 70/ | | 65/60 | 85/85 | 80/85 |
| L | 65/ | | 55/45 | 70/85 | 65/55 |
| PLASMA VISCOSITY (cp) | 1.79 | 1.80 | 1.84 | 1.75 | 1.81 |
| C-REACTIVE PROTEIN (g dm ⁻¹) | - | 0.050 | - | - | 0.024 |
| SERUM SULPHYDRYL LEVEL $\mu\text{M dm}^{-3}$ | 193 | 240 | 212 | 306 | 297 |

TABLE 3. Clinical and laboratory assessment of patient who did not repond to D-pen therapy (Case 3).

and D-pen treatment was stopped due to thrombocytopenia.

Monocyte ED₅₀ and IgG synthesis by these patients was monitored over the six month period. These parameters for the three case studies are presented in Fig. 27. The depressed IgG synthesis was steadily enhanced during the six months of D-pen therapy, irrespective of clinical improvement. The monocyte ED₅₀ does however appear to reflect clinical improvement, a gradual reduction in Ed₅₀ being achieved only in "Case 1". IgG synthesis and monocyte ED₅₀ data were placed into two groups (responders plus slow responders) and (non-responders) and are shown in Fig. 28 and Fig. 29 respectively.

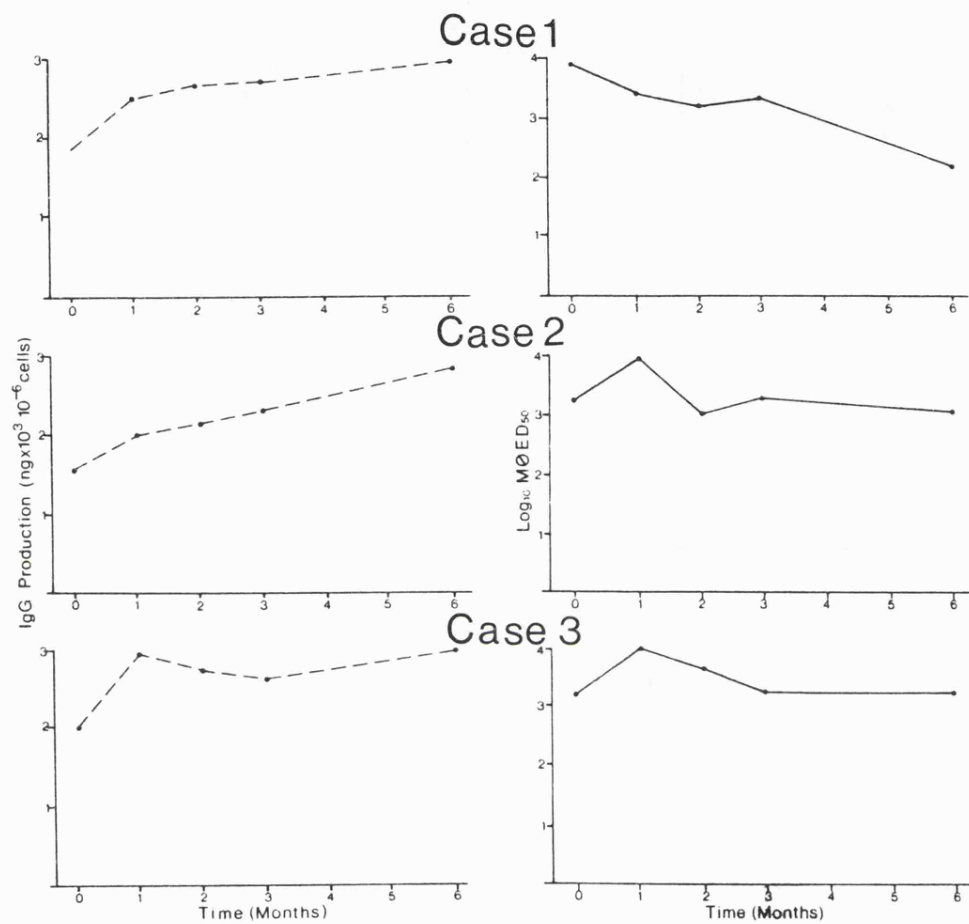


Fig. 27. IgG synthesis and monocyte ED₅₀ levels of the three case studies.

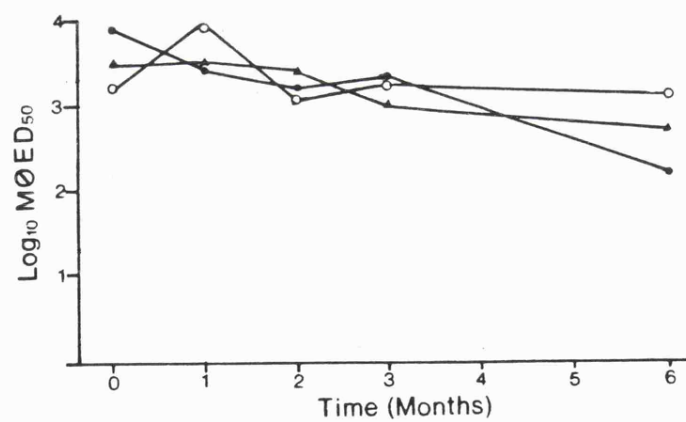
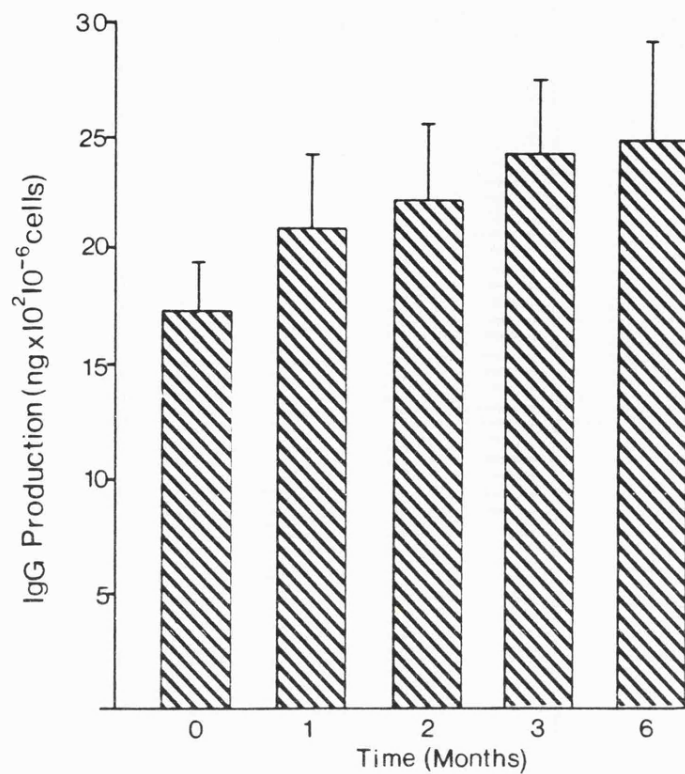


Fig. 28. IgG synthesis and monocyte ED₅₀ levels of the clinical responders and slow responders.

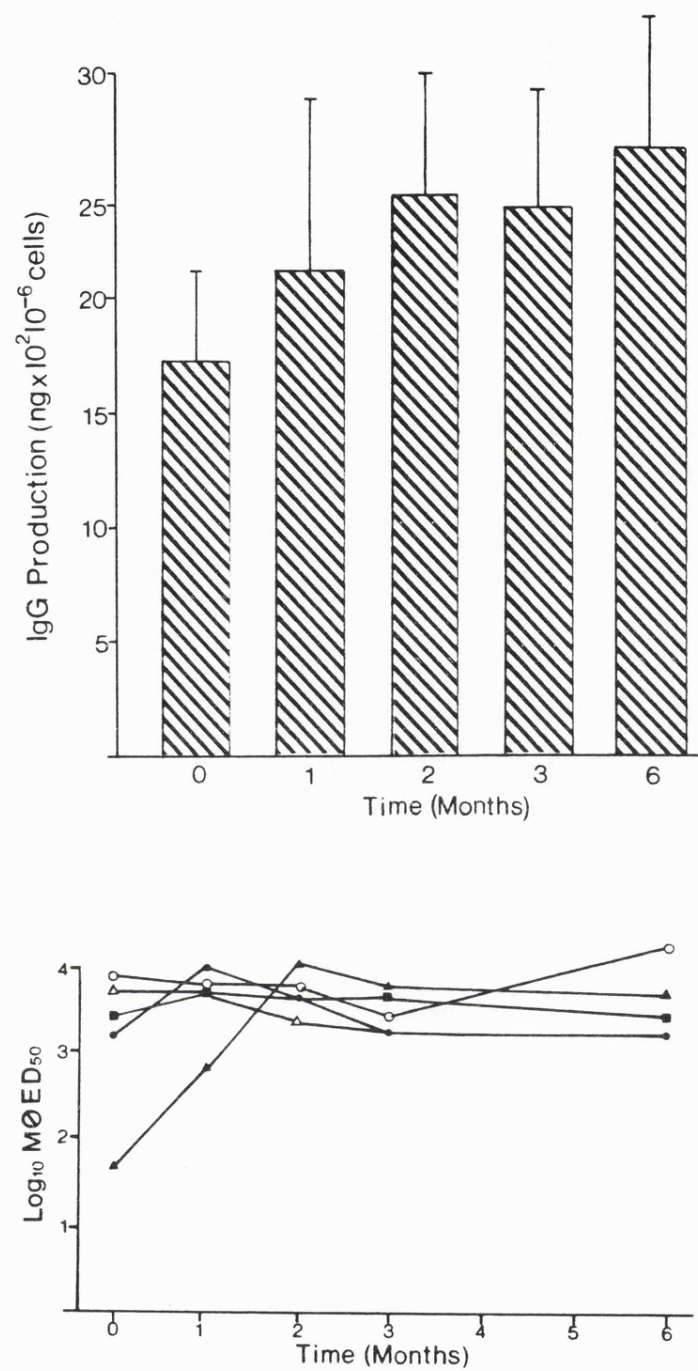


Fig. 29. IgG synthesis and monocyte ED₅₀ levels of the clinical non-responders.

3.1.11 Analysis of the action of 2-ME on monocyte-lymphocyte interactions

The stage at which 2-ME might exert its effect on monocyte-lymphocyte interaction was investigated. Mononuclear cell activation is mediated through complex interactions involving both cell:cell contact, necessary for antigen presentation, or through soluble mediators, for example the interleukins. Free SH groups may be necessary for any or all of these interactions.

3.1.12 Effect of 2-ME on interleukin production

Activated monocytes secrete IL-1 which acts on T-cells to stimulate the generation of IL-2 which in turn induces T-cell proliferation. As the IL-1 assay also detects IL-2 the IL-1 supernatants were also screened for IL-2 activity in a PHA blast culture system. All these results proved negative (data not shown). Fig. 30 shows IL-1 production by monocytes from three groups, healthy controls, RA-NSAID and RA D-pen. Monocytes from RA-NSAID patients secrete significantly more IL-1 than healthy cells in response to LPS ($P < 0.01$, Mann-Whitney U test). These results contrast markedly with the previous findings of an overall impaired accessory function showed by rheumatoid cells. The addition of 2-ME to the monocyte cultures did not alter IL-1 production. This is consistent with results in the final group, where RA D-pen

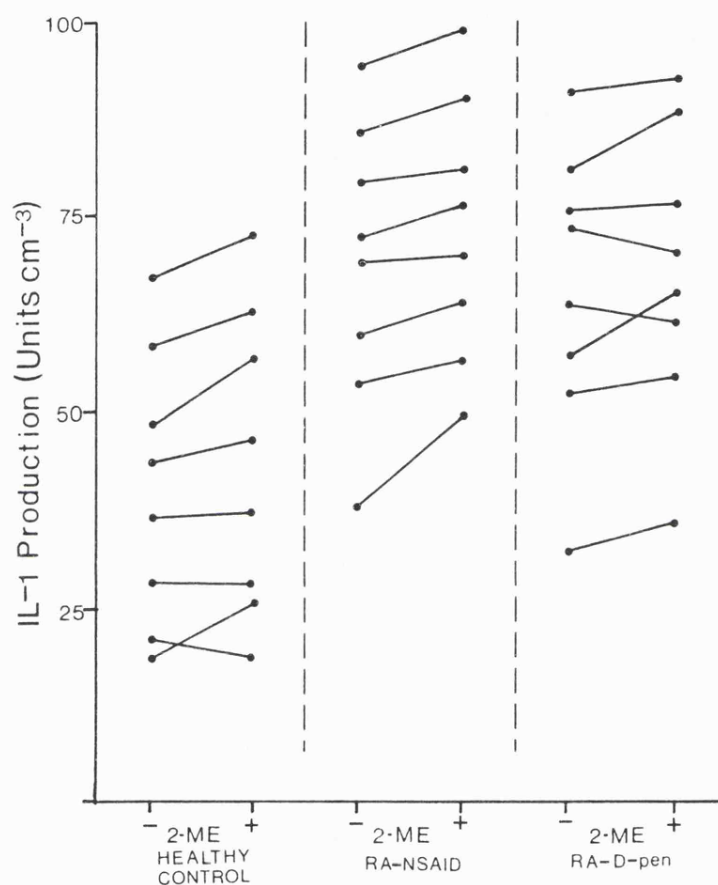


Fig. 30. Effect of 2-ME on IL-1 production. The dots represent IL-1 production (LAF) in arbitrary units in the presence or absence of 2-ME by monocytes from 3 groups, normal healthy controls, RA-NSAID and RA-D-pen ($n = 8$). The lines connect the dots which represent IL-1 production by cells from the same patient. IL-1 production is significantly greater by RA cells (RA-NSAID and RA-D-pen) than control cells ($P < 0.01$, Mann-Whitney U test). 2-ME had no effect on IL-1 production.

monocytes still showed elevated secretion of IL-1. The sulphydryl dependent step in the monocyte-lymphocyte interaction does not therefore appear to be IL-1 production. As it is IL-2 which actually induces T-cell proliferation the effects of SH groups on IL-2 production was also studied. IL-2 production was measured using cells isolated at the same time from the same blood samples as used for the IL-1 assays. There was no significant difference in IL-2 production between the three groups ($P > 0.05$, Mann-Whitney U test), although the values were very variable. The addition of 2-ME to the cultures did not have any significant effect ($P > 0.05$, Mann-Whitney U test) (Fig. 31). The production of interleukins does not appear to be sulphydryl dependent. The production of IL-1 and IL-2 did not correlate with each other (Fig. 32). Attempts to correlate levels of IL-1 and IL-2 production with clinical assessments of disease activity and also with biochemical parameters such as serum SH, CRP concentrations were unsuccessful and this data is presented in Table 4.

3.1.13 Studies involving antigen specific rat lymph node proliferation

Another possible stage at which 2-ME might exert its effect was antigen presentation. This would have proved difficult to study in a human system. Using facilities at the Lilly Research Centre antigen presentation was studied in a rat cell culture system. Rats sensitised with OVA were used

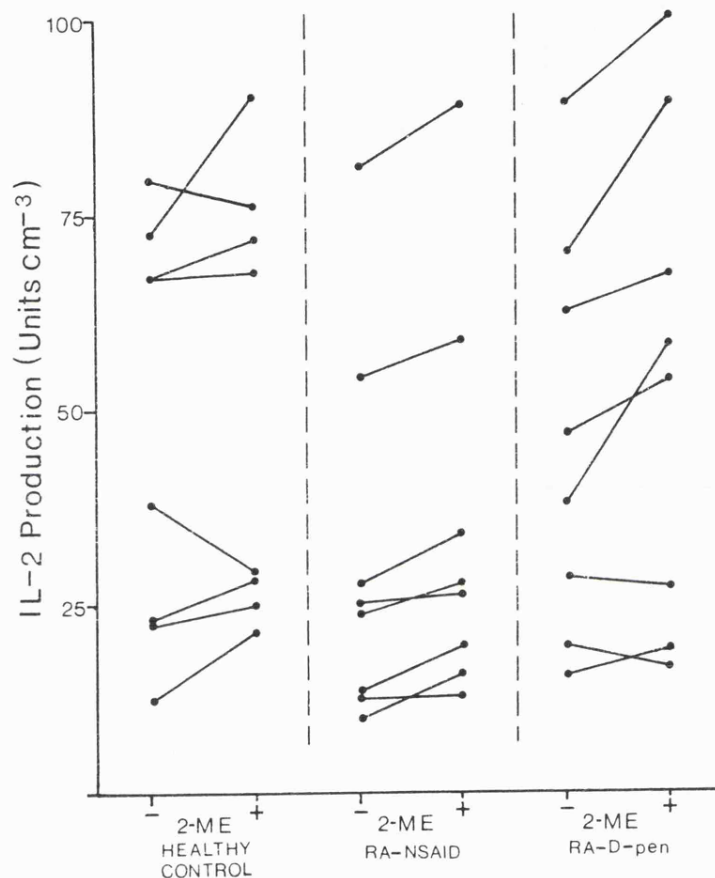


Fig. 31. Effect of 2-ME on IL-2 production. The dots represent IL-2 production in arbitrary units in the presence or absence of 2-ME from the same patients as Fig. 30, normal healthy controls, RA-NSAID, RA-D-pen (n = 8). The line connecting the dots which represent IL-2 production by cells from the same patient. There was no significant difference in IL-2 production from the 3 groups. 2-ME had no effect on IL-2 production.

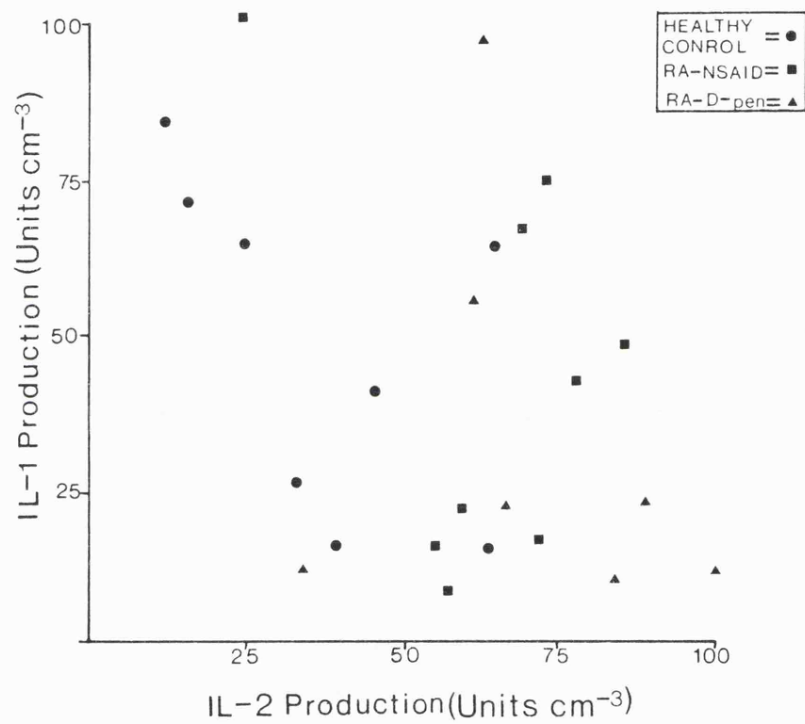


Fig. 32. Correlation of IL-1 and IL-2 production. The graph shows no apparent correlation between IL-1 and IL-2 from any of the 3 groups.

HEALTHY CONTROLS

| IL-1 Production (units 10^{-6} cells) | | IL-2 Production (units 10^{-6} cells) | | Serum -SH CRP ($\mu\text{M dm}^{-3}$) ($\mu\text{g cm}^{-3}$) | |
|--|------|--|------|---|------|
| -2ME | +2ME | -2ME | +2ME | | |
| 48.5 | 57.5 | 38 | 29.5 | 582 | < 10 |
| 19 | 26 | 79 | 76 | 487 | < 10 |
| 21 | 19 | 72.5 | 90 | 532 | < 10 |
| 43.5 | 47.5 | 12.5 | 21.5 | 558 | < 10 |
| 37 | 37.5 | 23 | 29 | 473 | < 10 |
| 71 | 74 | 67.5 | 73 | 505 | < 10 |
| 28.5 | 28 | 67.5 | 69 | 499 | < 10 |
| 59 | 63.5 | 22.5 | 23 | 547 | < 10 |

RA-NSAID

| IL-1 Production (units 10^{-6} cells) | | IL-2 Production (units 10^{-6} cells) | | Serum -SH CRP ($\mu\text{M dm}^{-3}$) ($\mu\text{g cm}^{-3}$) | |
|--|------|--|------|---|------|
| -2ME | +2ME | -2ME | +2ME | | |
| 39 | 51 | 12.5 | 13 | 368 | 55 |
| 60.5 | 65 | 25 | 26.5 | 240 | 30 |
| 80 | 81.5 | 13 | 19.5 | 365 | 46 |
| 69.5 | 70 | 81.5 | 90 | 197 | 38 |
| 95 | 97.5 | 28 | 34.5 | 295 | 57 |
| 54 | 57.5 | 24 | 28 | 375 | 18 |
| 72 | 77.5 | 55 | 58.5 | 262 | < 10 |
| 86 | 90.5 | 10.5 | 15.5 | 222 | 19 |

RA-D-PEN

| IL-1 Production (units 10^{-6} cells) | | IL-2 Production (units 10^{-6} cells) | | Serum -SH CRP ($\mu\text{M dm}^{-3}$) ($\mu\text{g cm}^{-3}$) | |
|--|------|--|------|---|------|
| -2ME | +2ME | -2ME | +2ME | | |
| 57.5 | 66.5 | 28 | 26 | 426 | 31 |
| 52.5 | 55 | 15 | 18.5 | 308 | 22 |
| 33 | 37 | 89.5 | 100 | 332 | < 10 |
| 64 | 62.5 | 19 | 17 | 406 | 40 |
| 73.5 | 71 | 62 | 67.5 | 477 | 114 |
| 76 | 76.5 | 70 | 90.5 | 275 | 15 |
| 91 | 92.5 | 47 | 53.5 | 418 | 37 |
| 81 | 89 | 38 | 58 | 263 | 27 |

Table 4. The relationship between IL-1, IL-2, serum SH level and CRP in the three patient groups.

in an antigen specific lymph node proliferation assay. Initial experiments were set up to study a similar reaction to those already described for human PBMC. This assay system was chosen because of initial observations regarding the cell surface thiols on rat cells. Preincubation of rat lymph node cells with PHMPSA did not inhibit their proliferation in response to OVA. Experiments were carried out to determine whether this was due to cell surface thiols being already blocked. Preincubation of the cells with 2-ME prior to preincubation with PHMPSA did result in inhibiting proliferation. The degree of inhibition was too inconsistent to group the data from several experiments together, but an example is presented in Fig. 33. This suggested the surface SH groups on the rat lymph node cells oxidised very quickly.

Further experiments with the OVA stimulated rat lymph node proliferation assay showed that once the lymph node cells had been totally depleted of macrophages cell proliferation was dependent on both added macrophages and 2-ME (Fig. 34a). Lymph node cells in the presence of antigen and macrophages only produced a small amount of proliferation (150 ± 75 CPM). The addition of 2-ME to the culture enhanced the cell proliferation to $16,003 \pm 6,552$ CPM (Fig. 34b). The physical separation of macrophages and lymphocytes resulted in the absolute necessity for the presence of a reducing agent in the culture system. Pre-incubation of cells in 2-ME did not have this effect (Fig. 34c). This data supported the initial

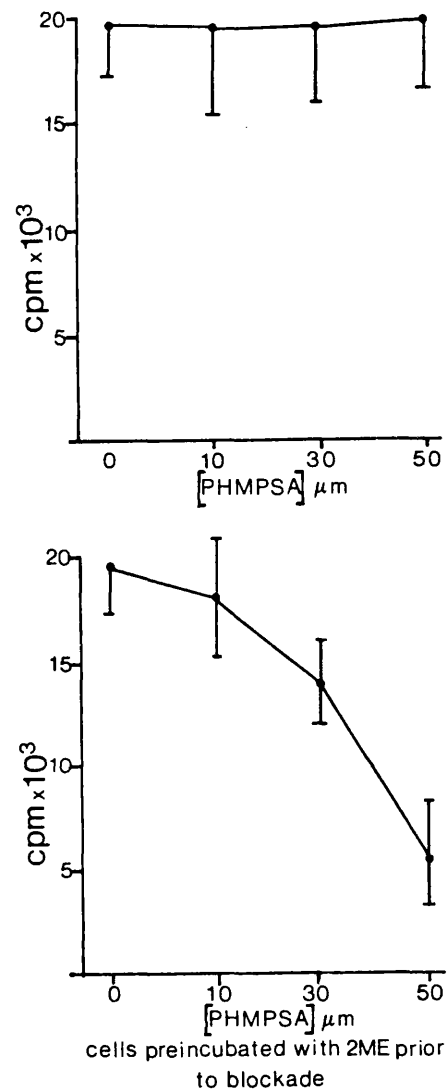


Fig. 33. The effect of PHMPSA on sensitised rat lymph node cells stimulated with OVA. Inhibition proliferation by PHMPSA is dependent on prior preincubation with 2-ME.

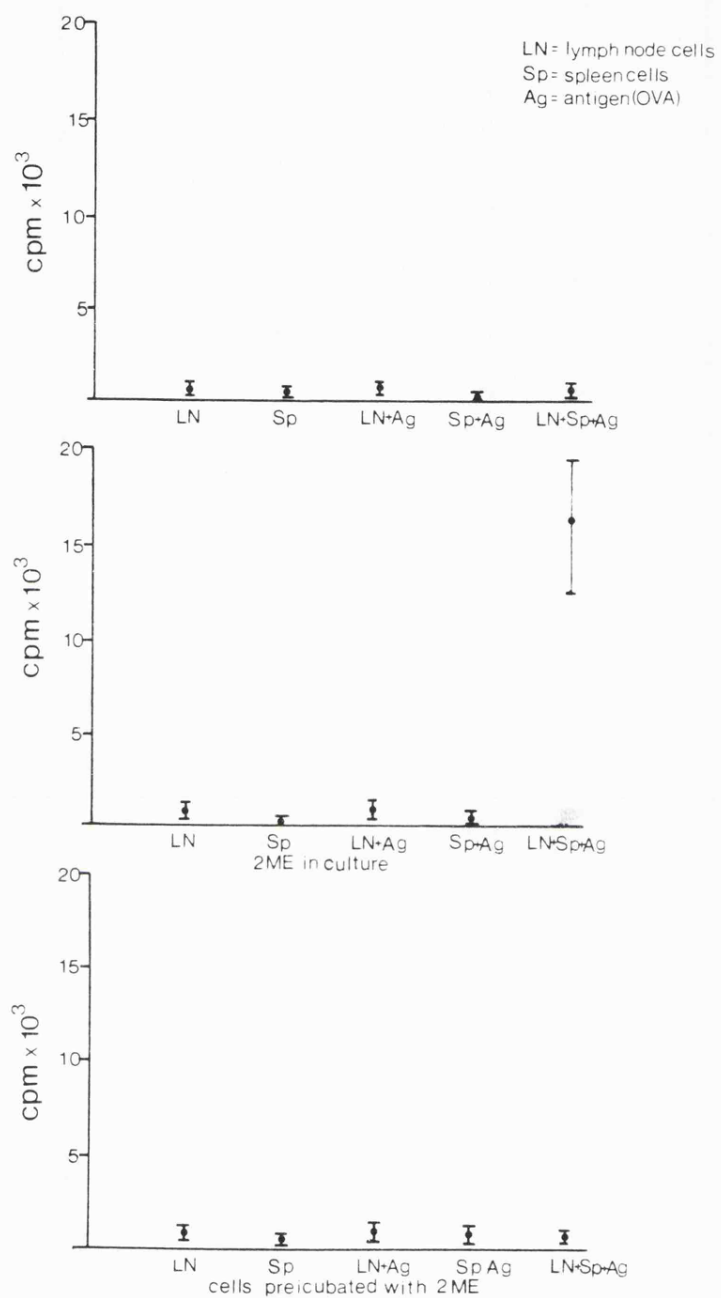


Fig. 34 a, b and c. The effect of 2-ME on cultures of adherent cell depleted rat lymph node cells. Adherent cell depleted lymph node cells are completely dependent on both added spleen cells and 2-ME.

| | -2ME | +2ME |
|--------------|---------------|-----------------|
| LN | 362 \pm 173 | 688 \pm 356 |
| LN + Ag | 96 \pm 43 | 500 \pm 126 |
| LN + Sp | 69 \pm 16 | 324 \pm 146 |
| LN + Sp + Ag | 427 \pm 132 | 16746 \pm 292 |
| Sp | 53 \pm 11 | 67 \pm 11 |
| Sp + Ag | 30 \pm 13 | 43 \pm 15 |

| | | |
|-----------------|--------------|------------------|
| PSp (+2ME) | 35 \pm 27 | 65 \pm 15 |
| PSp (+2ME) + LN | 233 \pm 81 | 14487 \pm 589 |
| PSp (-2ME) | 39 \pm 21 | 25 \pm 5 |
| PSp (-2ME) + LN | 91 \pm 36 | 13160 \pm 2156 |

Table 5. Rat lymph node proliferation

LN = Lymph node cells

Ag = Antigen

Sp = Spleen

PSp (+ 2-ME) = Spleen pulsed with antigen in the presence of 2-ME

PSp (- 2-ME) = Spleen pulsed in the absence of 2-ME

Results are expressed as the mean cpm (n = 3). In the absence of 2ME in the cultures there is no proliferation above background. Lymph node cells in the presence of both spleen cells and 2-ME showed enhanced proliferation (16746 \pm 292). The presence of 2ME was not necessary for antigen presentation. There was no significant difference (P > 0.05) in lymph node proliferation in cultures containing spleen cells pulsed in the presence, PSp (+ 2ME) or absence, PSp (- 2ME) of 2ME.

observations that the cell oxidised quickly and suggested that free SH groups were necessary for the monocyte and lymphocyte to physically interact.

Because this assay was a totally sulphhydryl dependent monocyte-lymphocyte assay it was used to determine whether the thiol dependent step was antigen presentation. There was no significant difference ($P > 0.05$, Mann-Whitney U test), whether the macrophages were preincubated with antigen in the presence ($14,487 \pm 589$ CPM) or absence ($13,160 \pm 2,156$ CPM) of 2-ME (Table 5).

Whilst free thiols did not appear to be needed for antigen presentation the data did show that rat cells are similar to human cells but oxidise much more rapidly.

CHAPTER FOUR

Discussion

Monocytes play a central role in modulating lymphocyte functions. Hewlett (1977) proposed that the accessory role of the monocytes was mediated by an SH dependent immunoregulatory molecule (MaSF) which was similar or identical to serum albumin. Although the results presented here show that serum albumin is essential for an immune response in vitro, the possession of a free SH group was not necessary.

Hall et al., (1984) have suggested that the free SH group on the albumin molecule may scavenge oxidative species, especially hydrogen peroxide, generated during the inflammatory response, thereby preventing widespread tissue damage. The low levels of serum SH found in RA patients may therefore reflect a persistent inflammatory response that is generating large amounts of peroxides. Under these conditions, the protective effect of the albumin may be insufficient to prevent cellular damage.

In serum an equilibrium between albumin and small molecular thiols such as cysteine would be expected. This equilibrium would also extend to cell membrane thiols. It would seem reasonable to suppose that whatever oxidises the serum SH groups would also block the cell surface SH groups on

the PBMC. An association between serum SH levels and the number of free SH groups on PBMC surfaces was found in this study. This finding supports the work of Lorber and Chang (1968) who found that the number of erythrocyte cell surface SH groups also correlated with the depression in serum SH levels. The results presented here show that cell surface thiols are necessary for a normal immune response. Chen and Hirsch (1972) suggested that these free SH groups are important at an early stage in PBMC activation. This is also supported by the findings of Chaplin and Wedner (1978), who found that the sulphhydryl oxidising agent diamide was only effective when added within 30-60 minutes of the mitogen. These results suggest that the maintenance of free sulphhydryl groups is important during the early induction of lymphocyte activation and indicates that an obligatory step or steps in the activation sequence may involve sulphhydryl interactions.

The work of Rosenstreich and Mizel (1978) showed that the reducing agent 2-ME synergises with very small numbers of monocytes to restore their accessory role in IgG synthesis. This suggests that free SH groups on the monocyte cell surface are necessary for their accessory function. In agreement with other workers (Rosenberg and Lipsky, 1981), PWM stimulated IgG synthesis by human lymphocytes is totally dependent on added monocytes. However, the results presented here show that blockade of surface SH groups on either the monocytes or lymphocytes abolished their ability to synthesise IgG when

recombined. This requirement for free cell surface SH groups for a normal immune response may explain the observed hyporesponsiveness of rheumatoid PBMC in vitro, since surface SH groups on these cells are partially blocked.

PBMC from patients with active RA were found to produce less IgG than normal cells when stimulated with PWM in vitro and this confirms data reported by other investigators (Alarcon et al., 1982; Patel and Panayi, 1982; Poikonen et al., 1982; Plater-Zyberk et al., 1983). However, normal IgG synthesis was observed with PBMC from patients treated with D-pen, and the addition of 2-ME to the rheumatoid PBMC enhanced the depressed levels of IgG synthesis to those produced by both the healthy and D-pen treated cells. As the 2-ME had little influence on IgG production in the latter two groups it is possible that the enhancing effect of 2-ME in vitro had already been achieved by D-pen in vivo.

The monocyte titration experiments demonstrate that monocytes from patients with active RA show an impaired accessory function in the PWM stimulated culture system when compared with normal cells. Similar results have been obtained in a recent study on a small group of RA patients, (Plater-Zyberk et al., 1983). These authors did not have a completely monocyte-depleted assay, so quantitation of the differences between rheumatoid and healthy cells was not

possible. The results presented here suggest that rheumatoid monocytes are as much as ten-fold less active as accessory cells than monocytes from normal controls. This contrasts with results from assays of monocyte phagocytosis in RA, which show normal cell function (Temple and Loewi, 1977; Bar-Eli, et al., 1980; Minty et al., 1983).

The accessory function of rheumatoid monocytes may be corrected in vitro by the addition of 2-ME (5×10^{-5} M) to the cultures. A similar improvement in monocyte accessory function seems to be achieved in patients following treatment with D-pen. Data from the monocyte titration curves suggest that the number of healthy monocytes that has to be added to obtain maximum IgG synthesis in vitro is 10^5 10^{-6} lymphocytes (Fig. 23). This is less than the proportion normally found in PBMC. In contrast no such maximum appears to have been reached in the assay containing cells from rheumatoid patients treated with NSAID. Thus it is possible that had more than 10^5 rheumatoid monocytes been added back to the system normal immunoglobulin synthesis might have been achieved.

These results therefore indicate that the impaired monocyte accessory function in patients with active RA is due to reversible blockade of surface SH groups, which will be re-exposed following the addition of 2-ME or by D-pen treatment. The PBMC surface SH groups are probably depressed in active RA

by oxidation and mixed disulphide formation with cysteine as are serum SH groups (Thomas and Evans, 1975) and probably also erythrocyte membrane thiols (Lorber and Chang, 1968). The depression of PBMC surface SH groups appears to affect certain cell functions, for example accessory function but not others, e.g. phagocytosis. It is suggested that cell surface SH oxidation may be responsible for some of the depressed PBMC functions displayed by rheumatoid cells in vitro.

The serial study of monocyte accessory function showed that enhanced IgG synthesis, in vitro, occurs rapidly following D-pen therapy regardless of clinical outcome. However, the monocyte ED₅₀ was reduced steadily only in the one patient who showed marked clinical improvement. The monocyte titration assay may therefore provide a means of monitoring RA patients during D-pen therapy. Further research is being undertaken to determine whether this assay may have any predicative value. The need for such a test is emphasised by the fact that only one patient of the fifteen studied showed marked clinical improvement and this patient was not suffering from a severe form of the disease.

D-pen and 2-ME are both thiol compounds and it is proposed that their mode of action is similar in that they act as reducing agents, restoring the free SH groups that are necessary for a normal immune response and which have been oxidised in RA. This hypothesis may seem to contradict the

explanation put forward by many workers that D-pen is an immunosuppressive drug (Maini and Roffe, 1976; Room and Maini, 1981; Lipsky, 1981). This conclusion has been reached from experiments in which cells have been treated in vitro with D-pen. These workers report that 0.1 mg cm^{-3} is required to inhibit lymphocyte proliferation and immunoglobulin secretion. Maini and Roffe (1976) have however shown that in whole blood cultures 1 mg cm^{-3} was needed to inhibit PHA stimulated lymphocyte proliferation whereas $1 \text{ } \mu\text{g cm}^{-3}$ and $100 \text{ } \mu\text{g cm}^{-3}$ D-pen augmented the response. Thus D-pen may have a biphasic effect similar to the effect of 2-ME on IgG synthesis presented here, augmenting the immune response in vitro at low concentrations and inhibiting it at high concentrations. The levels of D-pen measured in the serum of RA patients being treated with the drug are generally in the range $5 - 15 \text{ } \mu\text{g cm}^{-3}$, ten times less than that needed to inhibit PBMC responses in vitro. It is also important to remember that 90% of the D-pen is protein-bound. The work of Lipsky (1981) and Lipsky and Ziff (1982) is interesting in that, contrary to Room and Maini (1981), they found that copper was necessary to obtain the inhibitory effect of D-pen. Lipsky and Ziff (1982) proposed that this immuno-suppressive effect was due to the copper-chelating properties of D-pen. They suggest that a complex is formed in culture between the D-pen and the copper ion and this D-pen-copper complex is the inhibitory moiety. Lipsky (1984) proposed that the effect of D-pen in the presence of copper was inhibition of helper T-lymphocytes

involving the generation of hydrogen peroxide. A likely explanation for these apparently contradictory results is that whilst Room and Maini added $100 \mu\text{g cm}^{-3}$ of D-pen for the entire culture period, Lipsky (1978) and Lipsky and Ziff (1982) pre-incubated the cells with the drug in serum-free medium. A great deal more D-pen would be available than in the presence of serum, either in vitro or in vivo. The copper would act as a powerful catalyst for the oxidation of the cell surface SH groups on PBMC. The D-pen and copper might therefore act in the same way as PHMPA. Lewins et al., (1982) also report a dose dependent suppression of IgG production but found that the presence of D-pen was needed at the initial stage of the culture. This would confirm the results presented here that the free SH groups are essential for normal immunoregulation, but it is not realistic to conclude that the action of D-pen is immunosuppressive, but rather that at the levels found in the sera of D-pen treated RA patients is probably in fact the opposite, immunostimulatory. The work of Room and Maini (1981) is also interesting in that they reported that cells from rheumatoid patients were less susceptible to inhibition by D-pen than normal cells. This may well be due to the SH groups already being partially blocked.

Monocytes from rheumatoid patients treated with NSAID only secreted significantly higher amounts of IL-1 than did monocytes from healthy controls. This contrasts markedly with

the previous results of an overall depression in accessory cell function by rheumatoid monocytes. This enhanced IL-1 production may indicate that monocytes from RA patients are in a state of activation. The resultant immune and inflammatory responses induced by IL-1 may well contribute to the immunopathogenesis of RA, however it does not seem to be the SH-dependent reaction within overall monocyte accessory function. The addition of 2-ME to the cultures in vitro did not affect the enhanced IL-1 secretion. D-pen therapy did not affect IL-1 production in vitro. SH groups do not therefore appear to be involved in IL-1 production. Rheumatoid T cells did not respond to the IL-1 enhanced level by generating excessive amounts of IL-2. Indeed, IL-2 production by rheumatoid cells was in fact modestly lower than normal, which is in agreement with a recent report from Alarcon-Segovia et al. (1984). Again the addition of 2-ME in vitro or D-pen treatment in vivo had no significant effect on IL-2 production. IL-1 production did not, as might be expected, correlate with IL-2 production. This may support the possibility that IL-1 is not a single molecule and the IL-1-like activity present in the culture media may be different depending on the disease state of the donor's cells. The correlation of IL-1 with clinical assessment of disease activity and also with biochemical parameters such as serum SH and CRP failed to show any significant associations. The SH dependent step in the interaction between lymphocytes and monocytes does not therefore appear to involve the interleukins.

The experiments with rat lymph node cells showed that removal of adherent cells by passage down a Sephadex G-10 column abolished antigen stimulated lymphocyte proliferation. This is consistent with reports from others workers and with the work presented here. However, the response could only be recovered with added macrophages in the presence of 2-ME and not by pre-incubation of cells with 2-ME alone. The unresponsiveness of rat cells to PHMPSA blockade suggests that their SH groups oxidise very quickly, also a characteristic of rat serum SH groups (Butler et al., 1969). These results are interesting for two main reasons. First, they provided an assay system for determining whether free SH groups were necessary for antigen presentation. The results show that the presence of free SH groups on macrophages are not obligatory for antigen presentation. There was no significant difference in lymph node cell proliferation whether macrophages were pulsed with antigen in the presence or absence of 2-ME. Secondly, the results provided an insight into why cell surface SH groups are necessary for a normal immune response. The adherence of the macrophages to the Sephadex G-10 column, and the subsequent elution of the lymphocytes may result in the breaking of some physical interaction between the lymphocytes and macrophages involving the formation of disulphide bonds between SH groups on the two cell types. Because the SH groups auto-oxidise very quickly, the addition of 2-ME to the culture medium may be necessary to allow these bonds to be formed again for cell proliferation to take place.

Results obtained using rheumatoid monocytes and lymphocytes are consistent with this idea, since both cell types had to be treated with 2-ME before enhanced IgG synthesis was observed. This suggests that oxidised cell surface SH groups prevent a normal interaction between the monocytes and lymphocytes.

Lymphocytes interact physically with monocytes during the in vitro immune response to antigens. This interaction is manifested by a characteristic formation of cell clusters, (Werdelin et al., 1974). These cell clusters consist of a monocyte surrounded by lymphocytes. Cluster formation is also observed in mitogen stimulated cultures (Hawkes and Kennedy, 1984, personal communication). These workers report that IgG synthesis by PBMC stimulated with PWM was inhibited in a dose dependent manner by a mixture of D-pen and copper sulphate. This mixture also inhibited cell clustering stimulated by both PHA and PWM. Whether these observations have any relevance to the in vivo mode of action of D-pen or whether the drug is merely acting as a blocking agent at unrealistic levels is unclear. These findings are however consistent with the results presented here in that both suggest SH groups are involved in the physical interaction between lymphocytes and monocytes. Whilst this hypothesis explains the hyporesponsiveness found in in vitro stimulated PBMC, the situation in vivo is in fact the reverse. The nature of the disease is often one of hyper-activity of the inflammatory and immune responses. The answer may lie in the fact that

in vitro cultures employ PBMC. The situation in the synovium may be quite different. The macrophage lymphocyte interactions initiated by an unknown antigen may be locked together by the oxidative conditions in the synovium. This in turn would cause the production of oxidative species resulting in a positive feedback loop which may account for the uncontrolled inflammation and tissue destruction characteristic of RA. The free SH groups provided by D-pen treatment may simply act as a reducing agent to alter the oxidative conditions and end the positive feedback (Fig. 35). The delayed action of the drug may reflect a necessary build up of drug levels in tissue to achieve an effect. However, such is the uncertain nature of the mechanism by which lymphocytes and macrophages interact and the factors which regulate their responsiveness that any non-specific modulation of them must be somewhat unstable and unpredictable. There is the constant possibility that its beneficial effects may be reversed resulting in further defects in the immune system. Amongst the relatively high incidence of adverse reactions to D-pen are the production of auto-antibodies, auto-immune phenomena and auto-immune diseases other than RA. Jaffe (1979) has reported D-pen induction of pemphigus, myasthenia gravis, Sjogren's syndrome, Goodpasture's syndrome, systemic lupus erythematosus, polymyositis, immune complex nephropathy, thyroiditis, and haemolytic anaemia. Similar side effects have also been reported with other thiol drugs (Jaffe, 1980).

If the thiol containing drugs such as D-pen are the first of a new generation of antirheumatic drugs the future may lie in compounds which modulate SH dependent immune reactions in a more specific way, producing beneficial effects in more patients with fewer adverse reactions.

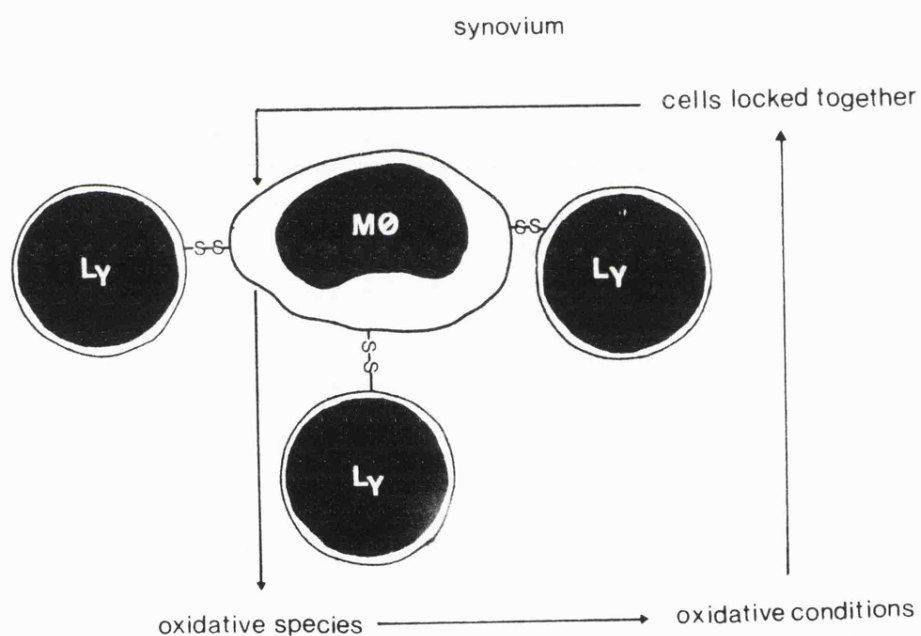


Fig35

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